The biology of biofilm bacteria from urinary tract catheters

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Meiner Familie

„Je mehr du gedacht, je
mehr du getan hast, desto
länger hast du gelebt.“

Immanuel Kant

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I. Abbreviations

%	percent
°C	Celsius
µ	micro
λ	Lambda
A	ampere
AUM	artificial urine medium
BCCM	Belgian Coordinated Collections of Microorganisms
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CAUTI	catheter associated urinary tract infection
CFU	colony forming units
CLED	cystine lactose electrolyte deficient medium
DNA	desoxyribonucleic acid
dNTP	deoxyribonucleotide
ddNTP	dideoxynucleotides
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPS	extrapolysaccharide
FESEM	field emmission scanning electron microscopy
Fig.	figure
g	gram
GC	gas chromatography
h	hour(s)
Hep2	larynx epidermoid carcinoma cell line
HZI	Helmholtz-Zentrum für Infektionsforschung
k	kilo
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	litre

LMG	Bacterial collection of the Laboratory for Microbiology of the Faculty of Sciences of Ghent University
LB	Luria-Bertani (medium)
M	molar
min	minute
MS	mass spectrometry
n	nano
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
sec	seconds
SEM	scanning electron microscopy
SSCP	single strand confirmation polymorphism
tab.	table
TAE	Tris base, acetic acid and EDTA buffer solution
TEM	transmission electron microscopy
TEMED	N,N,N',N'-Tetramethylethyldiamine
Tris	tris(hydroxymethyl)aminomethane
TSA	triptic soy agar
U	unit
UTI	urinary tract infection
V	volt
v/v	volume per volume
w/v	weight per volume

1. Introduction

1.1 Urinary tract infections

Urinary tract infections belong to the most frequent nosocomial infections with estimated 150 million patients per year, worldwide [Daschner *et al*., 1982; Ejaz *et al*., 2006]. About 80% of these infections are catheter associated. Bacteriuria is found for 5% of the patients after one month of catheter application [Garibaldi *et al*., 1974]. However, the majority of catheterized patients show no obvious symptoms, even the catheters get blocked by microbial colonisation after a certain time of carriage. Colonisation of the catheters with microorganisms can be detected in 53% of the cases [Bhatia *et al*., 2010].

1.2 Catheter associated urinary tract infections

Catheter associated urinary tract infections (CAUTI) constitute 40 percent of all nosocomial infections. About four million people each year receive an indwelling urinary tract catheter, and 5–20% of these patients will be diagnosed with a CAUTI [Gokula *et al*., 2004; Wong and Hooton, 1981]. Indwelling catheterization is commonly used in hospitals, intensive care units and nursing homes and increases the risk for CAUTI because the catheters can serve as the site of entry for opportunistic pathogens into the human body [Donlan and Costerton, 2002; Williams and Stickler, 2008].

CAUTI is defined as the new appearance of more than 10^3 colony forming units per mL of urine [Tambyah *et al*., 1999] and is rarely symptomatic [Tambyah and Maki, 2000]. Non-symptomatic/asymptomatic CAUTI means that this threshold limit is exceeded but the patient lacks symptoms of an infection. If CAUTI becomes symptomatic, the symptoms can range from mild to severe, fever, acute pyelonephritis, bacteremia, catheter obstruction, urinary stones, chronic interstitial nephritis and renal failure are possible

consequences [Warren, 1994]. Not only health of the patients is compromised but there is also an economic issue because costs of patients care increases immensely. Additionally, the antibiotic treatment is ineffective in most cases because bacteria causing CAUTI are in most cases organized in biofilms, which contributes to their antibiotic resistance [Jacobsen *et al*., 2008]. Biofilm-forming bacteria enter the human urinary tract either via the intraluminal route through the catheter lumen or via the surface of the catheter at the mucosal side. Host proteins deposited on the catheter surface allow the invading bacteria to adhere and initiate biofilm formation. In most cases CAUTIs are community acquired [Moore *et al*., 2002]. Often elderly catheterized patients suffer from permanent CAUTI. Eighty five percent of patients have more than two bacterial strains and 10% have more than five strains present [Stamm, 1991]. CAUTI biofilm infections are handled with different strategies. The first approach is to avoid initial contamination by aseptic techniques, second is to reduce microbial attachment. Thirdly, chronic CAUTI is usually treated with 5 to 10 days of antibiotic therapy [Trautner and Darouiche, 2004]. However, as known for other biofilm-associated infections, antibiotic treatment of asymptomatic CAUTI does often not cure the patients [Nicolle, 2005a]. Consequently, the last step is the removal of the catheter [Aslam, 2008; Trautner and Darouiche, 2004].

1.3 Community structure of catheter biofilms

Catheter biofilms have been reported to consist of two to five different species [Nicolle, 2005b; Tambyah *et al*., 1999] belonging to Gram-positive as well as Gram-negative bacteria. The most frequently isolated bacteria are Gram-negative Enterobacteriaceae, especially *Escherichia coli*, *Proteus* spp. and *Klebsiella* spp., as well as *Pseudomonas aeruginosa* and Gram-positives like Staphylococci and Enterococci. Most of the organisms originate from the patients skin or they are fecal contaminants [Daifuku and Stamm, 1984]. A recent culture-dependent study of 535 contaminated catheters revealed that the number of isolated species per catheter ranged between one and six.

The most frequently isolated bacteria were *Enterococcus faecalis* (294 strains), *E. coli* (213 strains) and *P. aeruginosa* (148 strains) [Hola *et al*., 2010]. Similar findings were obtained in a culture-independent analysis of Foley´s catheter biofilms using rRNA-based identification by Frank and coworkers [Frank *et al*., 2009].

Although indwelling catheters have been analyzed using culture-independent as well as culture-dependent techniques as mentioned before, there are a lot of open questions. While culture dependent analyses of bacterial biofilms allow quantification of species by viable cell counts, they do not cover the entire microbial diversity due to a majority of not culturable bacterial species. On the other hand, 16S rRNA gene based identification of bacteria provides full information on the species composition but only gains unreliable information concerning their quantitative distribution. Until today, no attempts have been made to link microbial diversity of CAUTI biofilms with the functional properties of individual organisms *in situ*. Studies on the transcriptional or the proteomic level only focused on individual species, using *in vitro* cultivation or animal models.

1.4 Phenotypic properties of bacteria from microbial communities on urinary tract catheters from patients without symptoms

Catheters are typical devices *via* which environmental microorganisms can enter the human urinary tract [Williams and Stickler, 2008]. Microorganisms usually colonise medical devices as catheters by forming biofilms [Donlan and Costerton, 2002]. The growth of a bacterial biofilm on a urinary catheter progresses according to a well-defined sequence of events (Figure 1). First, bacteria attach to the urinary catheter and initiate a biofilm by forming a sheet of organisms which coats the catheter. Next, they secrete an extracellular matrix often composed of exopolysaccharides (EPS) in which they become embedded [Stamm, 1991]. Catheter application is an invasive process causing tissue damage which results in a typical immune response. Initially,

for most patients these processes remain without obvious symptoms. However, the basis for severe infections might be laid. Therefore, it is discussed that biofilm formation represents a major cause for CAUTI. If a CAUTI becomes symptomatic this can result in fever, pyelonephritis or sepsis [Sedor and Mulholland, 1999]. Furthermore, parts of the biofilm can detach from the biofilm and colonise the mucosal site of the bladder (Figure 1.6).

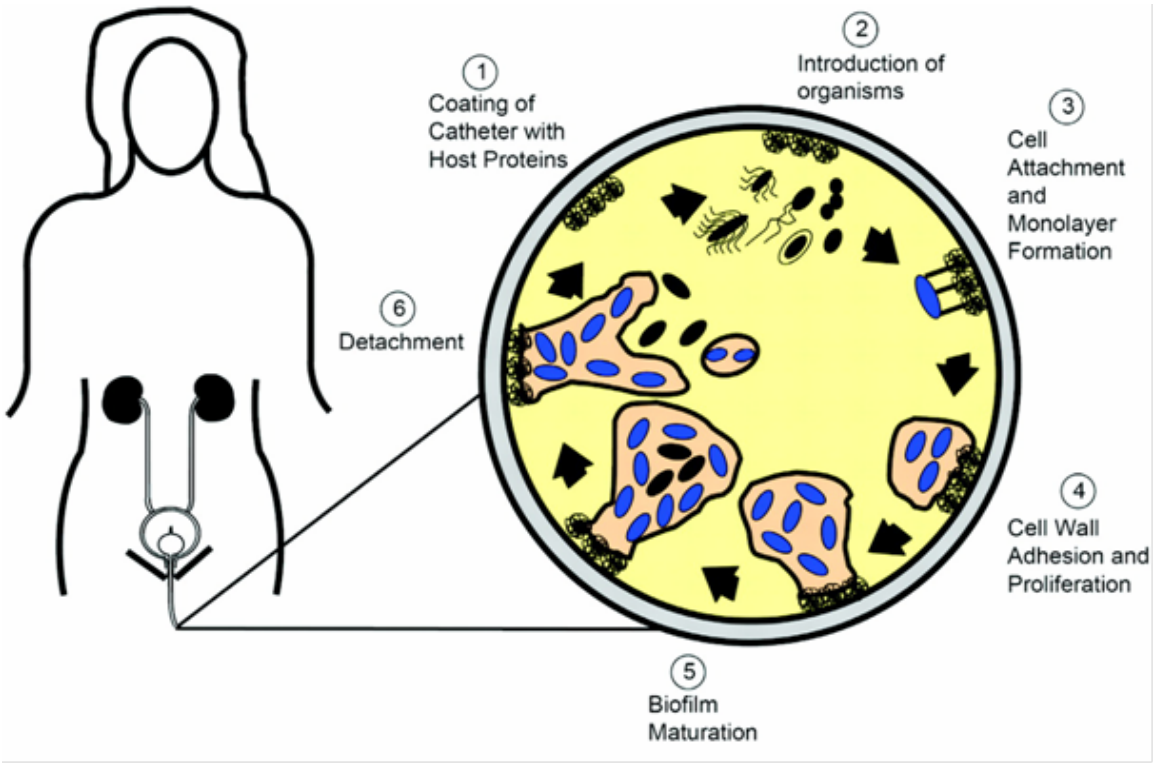

Figure 1: Pathogenesis of biofilm formation on urinary catheters during CAUTIs (Jacobsen, Stickler, Mobley & Shirtliff, 2008)

Several bacterial species were identified in the context of CAUTI and other urinary tract infections. The most frequent isolated bacteria from catheters are *E. coli* (60%), *Klebsiella* sp., *Staphylococcus* sp. and *Enterococcus* sp. [Bhatia *et al*., 2010]. All of these are commensal or mutualistic bacteria. Biofilm formation of urinary tract isolates was examined intensively for strains of *Acinetobacter baumannii*, *E. coli*, *Proteus mirabilis* and *Enterococcus faecalis* [Cernohorska and Chvilova, 2011; Giridhara Upadhyaya *et al*., 2010; Pour *et al*., 2011; Tapiainen *et al*., 2011]. However, investigations were usually carried out at the single species level.

Hemolysis was found to be an important virulence factor in Enterococci and Enterobacteriaceae during urinary tract and other nosocomial infection [Arthur *et al*., 1989; Coque *et al*., 1995]. DNase activity can be involved in virulence processes, e.g. for group A Streptococci or *Enterococcus* sp. [Elsner *et al*., 2000; Sumby *et al*., 2005]. Urea hydrolysis performed by *Proteus* sp. and other urease producing organisms leads to crystalline precipitations on the catheter surface and thereby causes complicated CAUTI in some cases [Kunin, 1989]. Furthermore, the secretion of proteases is known to play an important role in virulence and biofilm formation [Snell *et al*., 1978; Tielen *et al*., 2010].

Antibiotic resistance of biofilms is mediated by the slow growth of the bacteria in the biofilm, the diffusion barrier of the EPS matrix and organism specific resistance strategies (Donlan, 2002). In order to develop alternative anti-biofilm strategies it is mandatory to identify the microorganisms involved in bacterial colonisation of Foley´s catheters and to analyse their phenotypic and biochemical properties. So far, most of the biofilm originated CAUTI microorganisms were analysed from patients treated in hospitals. However, urinary tract associated biofilms in catheters of non-infected patients have so far been neglected. Nevertheless, these biofilms might provide the basis for subsequent severe infections.

1.5 The genus *Myroides* in the context of infection

The genus *Myroides* consists of six different species. The first isolation of a strain of *M. odoratus* (formerly *Flavobacterium odoratum*) was in 1923 [Stutzer, 1923]. Due to 16S rRNA gene analysis the genus *Flavobacterium* was reorganized and the species *Flavobacterium odoratum* was split up to *M. odoratimimus* and *M. odoratus* [Vancanneyt *et al*., 1996]. Other species of the genus are *Myroides pelagicus* [Yoon *et al*., 2006]*, Myroides phaeus* [Yan *et al*., 2012], *Myroides marinus* [Cho *et al*., 2011] and *Myroides profundi* [Zhang *et al*., 2008]. Another recommended species, *M. injenensis* has only been described insufficiently by genome determination [Kim *et al*., 2012a].

Myroides spp. are described as aerobically living rods that are able to consume various carbon sources. Cells grow up to 0.5 x 1-2 µm [Yan *et al*., 2012]. Extended cells of *M. odoratus* of up to 12 µm occur in nutrient broth [Vancanneyt *et al*., 2011]. Spores are not formed. *Myroides* spp. are non-motile. Colonies produce a fruity odor on nutrient rich medium. Nitrate is not reduced. Cytochrome *c* oxidase, gelatinase and catalase are produced. Growth occurs between 18 and 37 °C [Vancanneyt *et al*., 2011].

Two of them, namely *M. odoratimimus* and *M. odoratus* (formerly *Flavobacterium odoratum*) were frequently isolated from clinical sources. *Myroides* isolates have been implicated in the context of soft tissue infection, septic shock and pneumonia, urinary tract infections, cellulitis, central venous catheter- associated bloodstream infection and bacteremia [Bachmeyer *et al*., 2008; Benedetti *et al*., 2011; Douce *et al*., 2008; Green *et al*., 2001; Ktari *et al*., 2012; Maraki *et al*., 2012]. Although frequently isolated from human sites, a natural habitat as commensal or pathogens for *Myroides* spp. is not known. They are proposed to colonize soil and seawater but extended studies are still missing. A good review about case reports and *Myroides* infections has been given by Benedetti and coworkers in 2011 [Benedetti *et al*., 2011]. *Myroides* spp. account for approximately 0.2% of all clinical isolates of non-fermentative, Gram-negative bacteria [Pickett *et al*., 1991]. However, the contaminating source remains unclear yet but water in the hospital environment is suspected [Hugo *et al*., 2006]. Moreover, some strains isolated from dairy sources and fish processing sites are supposed to be potential food spoilage organisms with thermostable proteases and lipases [Hugo *et al*., 2006].

1.6 Aim of the study

1. Until now, the microbial communities on urinary tract catheters of patients without symptoms are poorly understood. Consequently, the structure of microbial communities present on urinary tract catheter biofilms directly derived from the patient was to be investigated using DNA- as well as protein-based technologies. Information of 16S rRNA gene sequences should provide a deep insight into the phylogenetic structure of involved bacteria. The metaproteomic approach allows a semi-quantitative evaluation of the bacterial community composition together with information about the physiology of different phylogenetic groups present in the mixed catheter biofilms. Solving the structure and function of the microbial community is mandatory for future avoidance strategies of catheter associated urinary tract infections.

2. Urinary tract catheter biofilms derived from patients without symptoms might provide a reservoir for subsequent severe infections. Therefore, catheter derived bacteria from an urologist practice were to be analyzed for their colonisation and pathogenic potential. Community acquired bacterial strains isolated from Foley´s catheters removed from elderly asymptomatic patients from an urologist practice were to be identified by their 16S rRNA gene sequence. Their ability to hydrolyze urea and extracellular proteins and to produce hemolysin was to be tested. Furthermore, bacterial twitching, swimming and swarming capacity were to be determined. The biofilm formation potential was analysed. Moreover, the antimicrobial resistance to various different commonly used antibiotics via the disc diffusion method will be determined. Results were discussed in the context of urinary tract infections with focus on CAUTI.

3. Almost nothing is known about the biology of *Myroides* spp. in general and in the context of catheter associated urinary tract infection. Therefore, the isolate *Myroides* sp. A17 had to be characterized for its virulence potential and adaptations to the urinary tract. Moreover, the species affiliation had to be clarified using different taxonomic approaches.

2. Material and Methods

2.1 Sample Collection

Ninety two urinary tract catheters from patients with asymptomatic UTI were provided by a general practitioner in a surgery unit in Kassel, Germany. Samples were collected after catheterization for 28 days. Catheter tips were stored at 4 °C and DNA was extracted immediately after reception. A total number of 44 catheters were analyzed by single strand conformation polymorphism (SSCP) analysis. Five catheters were chosen for subsequent metaproteomic analysis. Out of these five catheters removed from four male and one female patient were analyzed. Patient's data are listed in Table 1 appendix. Catheter tips were cut off with a sterile scalpel; biomass was scrubbed from the surface aseptically and transferred to a tube for genomic DNA extraction.

2.2 Isolation of bacteria

Ninety two catheters from patients were supplied by the Physican Center for Urinary tract diseases, surgery unit in Kassel, Germany. All patients were asymptomatic, that means without obvious symptoms like pain or fever for the patient and without symptoms detectable for the urologist using standard examination procedures. Catheter tips were removed with a sterile scalpel; biomass was scrubbed aseptically from the surface, suspended in phosphate buffered saline and plated onto tryptic soy agar (TSA), (Roth, Karlsruhe, Germany), Difco MacConkey agar (Becton Dickinson, Le Pont de Claix France), CLED (Roth, Karlsruhe, Germany), Corynebacterium agar (DSMZ medium 53; casein peptone, tryptic digested 10.0 gL^{-1}, yeast extract 5.0 gL^{-1}, glucose 5.0 gL^{-1}, NaCl 5.0 gL^{-1}, agar 15.0 gL^{-1}, pH 7.2) and Luria-Bertani (LB agar). Agar-plates were incubated at 37 °C for 24 h. Colonies were picked

and transferred to fresh agar plates. Culture purity was checked macroscopically and microscopically.

Both *Myroides* strains A17 and A21 were isolated from urinary tract catheter biofilms derived from a 91 year old female outpatient. Biofilm mass was scraped from the outer catheter surface suspended in phosphate buffered saline and plated on tryptone soy agar (Roth, Germany). Agar-plates were incubated at 37 °C for 24 h. Single colonies were transferred to fresh agar after 24 h and further incubated. Monoculture purity was checked macroscopically and microscopically. Bacteria were stored in glycerol stocks at -80 °C. For further experiments cells were grown on LB (agar) at 37 ° C overnight. The reference strains *M. odoratimimus* (LMG 4029^T) and *M. odoratus* (LMG 1233^T) originated from BCCM/LMG bacteria collection were cultivated on LB and TSA, respectively.

Human laryngeal epithelial cell line HEp-2 (ATCC CCL23) was cultivated as described previously [Molinari *et al*., 1997].

2.3 Identification of bacteria

2.3.1 16S rRNA gene sequencing

Bacteria were identified *via* 16S rRNA gene sequencing and subsequent bioinformatic analysis. Genomic DNA was extracted from the cultured bacteria using the FastDNA Spin Kit for soil (MP Biomedicals, Strasbourg, France) after instructions of the manufacturer. For this purpose cells were lysed for 45 s at a speed of 5.5 m/s in a FastPrep24 Instrument (MP Biomedical, France). Subsequently, isolated strains were identified by 16S rRNA gene sequencing. The corresponding PCR was performed using the primers 27F and 1492R (position in the *E. coli* 16S rRNA gene, 27f: GAG TTT GAT CCT GGC TCA G and 1492r: AGA AAG GAG GTG ATC CAG CC) [Lane, 1991]. The DNA primers used in this study were synthesized by MeTabion, Martinsried, Germany. Reaction mixtures contained ThermoPol buffer, desoxynucleoside triphosphates (20 µM of each dNTP), 0.5 µM of

each primer, 2.5 U of DNA polymerase (ThermoTaq Polymerase, NEB, Ipswich, USA) and 100 ng genomic DNA. Cycle conditions for the reactions were initial denaturation at 95 °C (2 min), 28 cycles of 95 °C (20 s), 52 °C (2 min), and 72 °C (2 min) with a final extension at 72 °C (6 min). PCR Products were purified (Qiaquick PCR Purification Kit, Qiagen, Hilden, Germany). The DNA sequence of the PCR products was determined using the BigDye ® Terminator v1.1 Cycle Sequencing Kit and the 310 genetic analyser (both Applied Biosystems, Foster City USA) according to the instructions of the manufacturer**.** For sequencing reaction primers 27F, 1492R and Com1f were used [Schmalenberger *et al*., 2001]. Identification of the determined 16S rRNA gene fragment was performed using BLAST search of the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [Altschul *et al*., 1990]. The 16S rRNA gene sequences of uncultured and environmental isolates were excluded from the search to relate the isolates from this study to the next related species. Nine representative members of found bacteria were selected and subjected to further characterization studies. Identified bacteria were stored in glycerol stocks at -80 °C. For further experiments cells were inoculated in Luria–Bertani (LB) broth, incubated at 37 °C for 24 h.

2.3.2 Single strand confirmation polymorphism

Extraction and Purification of DNA

Total Genomic DNA from the catheter biofilm was extracted and purified using the FastDNA Spin Kit for soil (MP Biomedicals, France). Cells were lysed for 45 s at a speed of 5.5 m/s in the FastPrep24 Instrument (MP Biomedical, France).

PCR Amplification of 16S rRNA Genes

PCR was performed using the primersCom1f (CAG CAG CCG CGG TAA TAC) and Com2r-Ph (CCG TCA ATT CCT TTG AGT TT - Pho). For a subsequent single-strand digestion Com2-ph was phosphorylated [Schwieger and Tebbe, 1998]. The primers used in this study were synthesized by Metabion, Germany. Reaction mixtures contained 1x ThermoPol buffer, desoxynucleoside triphosphates (20 µM of each dNTP), 0.5 µM of each primer, 2.5 U/100 µl of TaqDNA polymerase (NEB, USA) and 1 µl genomic DNA- solution (50 ng). Cycle conditions for the reactions were initial denaturation at 95 °C (3 min), 30 cycles of 94 °C (50 s), 50 °C (50 s), and 72 °C (90 s) with a final extension at 72 °C for 10 min.

Agarose Gel Electrophoresis, PCR Purification and Lambda Exonuclease Digestion

Quality and size of PCR Products were analyzed by agarose gel electrophoresis.. Therefore, an aliquot of the PCR reaction was mixed with 5x GelPilot Loading Dye (Quiagen, Germany) and loaded onto an agarose gel (1% in TAE: 40 mM Tris, 20 mM acetic acid and 1 mM EDTA). For size control, the GeneRuler DNA Ladder Mix (Fermentas, Germany) was applied. Agarose gels were stained for 10 min. in 0.1% V/V ethidium bromide and visualized under UV light (312 nm). PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, Germany) according to instruction of manufacturer. To generate single stranded DNA fragments the Lambda exonuclease und buffer (NEB, USA) were applied. This enzyme catalyzes the removal of 5´ mononucleotides from duplex DNA. For this reaction, 10 µg of PCR products were mixed with 40 µl 10x reaction buffer and 0.5 U Lamda exonuclease. Volume was filled up to 40 µl with dest. water. Resulting single stranded fragments were dried under vacuum and suspended in 5 µl loading dye (95% formamide, 10% NaOH, 0.25% bromophenol blue and 0.25% xylen

cyanol FF). Before loading onto the SSCP gel, samples were denatured for 3 min. at 95 °C and cooled on ice.

SSCP gel electrophoresis

Single-strand-conformation polymorphism is a culture independent, PCR-based fingerprinting method for studying microbial communities [Su *et al*., 2012]. Using this technique, single stranded DNA is electrophoretically separated in dependency of the size of the different secondary structures of the nucleic acids. These secondary structures are due to sequence differences. SSCP profiles were generated and visualized as described elsewhere [Dohrmann and Tebbe, 2005; Schwieger and Tebbe, 1998; Tebbe *et al*., 2001]. Separation of the fragments was performed at 17 °C for 17 h and 400 V and 16 mA. Running buffer TBE was composed of 890 mM Tris-HCL (pH 8.0), 890 mM boric acid and 20 mM EDTA. DNA Molecular Weight Marker (Roche, Germany) was applied to both outer lanes of the SSCP gel. SSCP gels were silver-stained as described by Bassam and coworkers [Bassam *et al*., 1991].

Extraction, re-amplification and sequencing of DNA from silver-stained SSCP profiles

Bands of the SSCP profiles were excised with a sterile razor blade, and the single-stranded DNA of these bands was eluted for 10 min at 95 °C in 50 µL “crush and soak” buffer (500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA (pH 8.0) and 0.1% sodium dodecyl sulphate) [Sambrook and Russel, 2001]. The single stranded DNA fragments were recovered by PCR with the Com-primers in a 25 µL volume and the products were purified as described above. The re-amplified DNA molecules recovered from bands of the community profile were used for DNA sequencing [Dohrmann and Tebbe, 2005].

DNA Sequence analyses

For DNA sequencing, Com1 Primer was used in combination with the recommended BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA). Primer, template concentrations, reagents and the purification of the sequencing reaction products were selected and processes according to the instructions of the manufacturer. Conditions for the sequencing reaction were as follows: initial denaturation at 96 °C for 60 s, followed by 25 cycles of 96 °C for 30 s, 50 °C for 15 s and 60 °C for 4 min. Sequence were obtained by the automated sequencer ABI Prism 310 using 310 Genetic Analyzer Capillary (47 cm). Chromatograms of the sequencing reactions were analyzed using Sequence scanner v1.0 (ABI). Bioinformatic tools for the alignments and database identifications of the consensus sequences were carried out using NCBI-BLASTn search and EzTaxon.org [Chun *et al*., 2007; Zhang *et al*., 2000]. Uncultured and environmental isolates were excluded.

2.4 Phenotypic characterization of catheter isolates

2.4.1 Biofilm formation assay

Biofilm formation was quantified as described by Stepanovic and coworkers in 2007 [Stepanovic *et al*., 2007]. For this purpose, isolated strains were cultivated in LB or artificial urine medium (AUM) using microtiter tissue plates (cellGrade, Brand, Wertheim, Germany) in a microtiter plate incubator at 37 °C for 24 h. The obtained cultures were decanted carefully and washed three times with phosphate buffered saline (PBS: NaCl 8.01 gL-1, KCl 0.20 gL^{-1}, Na_2HPO_4 x 2 H_2O 1.78 gL^{-1}, KH_2PO_4 0.27 g^{L-1}, pH 7.4). The resulting biofilm layer was air-dried and stained with ethanol dissolved crystal violet. Biofilm growth was quantified by measuring OD at 595 nm [Stepanovic *et al*.,

2007]. The mean value of six independent replicates was calculated and background values were subtracted. To ensure that cells were enclosed in the stainable material biofilms were grown using the same protocol; biofilms were suspended in PBS and plated on LB agar plates. To mimic iron limiting conditions cells were cultivated in AUM which was supplemented with 0.2% glucose [Brooks and Keevil, 1997].

2.4.2 Urease activity test

Urease hydrolysis was monitored in microtiter tissue plates (cellGrade, Brand, Wertheim, Germany). Overnight cultures were diluted in 0.9% NaCl to a density according to McFarland standard of 1. Cells were grown in Urea Broth (Fluka/Sigma-Aldrich, St. Louis, USA) at 37 °C for 24 h. In the case of urease activity the cleavage of urea liberates ammonia, which is indicated by a color change of the pH indicator (phenol red) from yellow (pH 6.8) to red to pink-red (pH 8.1). Optical density was measured at 595 nm to quantify the reaction. The mean value of six independent replicates was calculated and background values were subtracted.

2.4.3 Hemolysis, DNase and Protease activity and motility tests

Test agar for protease contained 5% (w/v) skim milk powder dissolved in separately sterilized Caso agar (both Difco Laboratories, Detroit, MI, USA). Hemolytic activity was tested on Columbia Agar with 5% (v/v) sheep blood (Becton Dickinson, Le Pont de Claix France). Overnight cultures were inoculated to the agar plates and incubated aerobically at 37 °C up to 72 h according to the manufactures instructions. Hemolytic positive strains showed a transparent halo around the colonies. DNase was tested on DNase test agar plates with methyl green according to the manufacturer's

instructions (Becton Dickinson, France). Bacteria excreting DNase turned the green color of the agar plate around the colonies into yellow.
Motility agar plate assays for the determination of bacterial swimming, swarming and twitching motility were performed as described previously [O'Toole and Kolter, 1998; Rashid and Kornberg, 2000; Tielen *et al*., 2011].

2.4.4 EUCAST disc diffusion test for antimicrobial susceptibility

Antibiotic resistance testing was performed using the disk diffusion method as described before [Andrews, 2001; EUCAST, 2000] on Mueller-Hinton Bouillon (Sifin, Berlin, Germany) supplemented with 15 g/l agar. Overnight cultures (on Mueller-Hinton-Agar) were diluted in 0.9% (w/v) NaCl to a density according to MacFarland standard of 0.5. Discs with a diameter of 9 mm (Macherey-Nagel, Germany) were inoculated with the different antibiotics and placed onto the agar plates. Used antibiotics and dilutions are given in Table 1. Dose ranges for each antibiotic were calculated according to common MICs for each species and antibiotic [Andrews, 2001]. After incubation of the plate with the discs for 18 h at 37 °C zone diameters around the discs were measured. All tests were performed in triplicates of three independent experiments. Cut off values for detection of resistance were calculated according to EUCAST Clinical Breakpoint Table version 1.3. (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/EUCAST_breakpoints_v1.3_pdf.pdf).
Additionally, antibiotic resistance of *Myroides* sp. A17 was tested in LB broth in order to find out an appropriate antibiotic for the infection studies. Therefore, antibiotics were tested in serial dilutions up to 250 µg/ml. Apart from the already mentioned antibiotics, additional antibiotics were: chloramphenicol, streptomycin, carbenicillin, rifampicin, erythromycin, neomycin and spectinomycin (all Sigma- Aldrich, USA; Roth, Germany or Hexal, Germany).

Table 1: Preparation of antibiotic solutions and ranges for MIC determinations (Andrews, 2000)

Antibiotic	class	solvent/diluent	concentration [mg/L]	supplier
Ampicillin (sodium salt)	ß-Lactams	water	12,5-200	Roth (Karlsruhe, Germany)
Cefixim	Cephalosporin	water	5-75	Sigma-Aldrich (St. Louis, USA)
Ciprofloxacin	Fluorochinolone	water	1.25-20	Fluka/Sigma-Aldrich (St. Louis, USA)
Cotrimoxazole	Sulfonamide	water	160/32-20/8	Hexal (Holzkirchen, Germany)
Gentamicin (-sulfate)	Aminoglycoside	water	0.25-8	Roth
Levofloxacin	Fluorochinolone	water	0.1-32	Sigma-Aldrich
Nitrofurantoin	Nitrofuran	DMF	32-260	Sigma-Aldrich
Tobramycin (-sulfate)	Aminoglycoside	water	1.25-20	Sigma-Aldrich

2.5 Bioinformatic data processing

2.5.1 Statistical analyses

The frequency of a joint presence of two bacteria species (data derived by PCR SSCP Analysis) is determined from the data set resulting from all SSCP analysis for *Aerococcus* sp., *Proteus* sp., *Pseudomonas* sp., *Enterococcus* sp. and *Escherichia* sp., which is calculated by using the open source programming language R (R Development Core Team 2006 R: A Language and Environment for Statistical Computing Online at www.R-project.org). It is a software environment for statistical computing and graphics and available for all operating systems.

The total number of catheters with bacterium A (#(A)) in proportion to the catheters with the bacteria A and the bacteria B (#(B ∧ A)) are calculated as follow:

$p(B|A) = \#(B \wedge A)/\#(A)$

with

$\#(A) \in \mathbb{N}$ and $\#(B|A) \leq \#(A)$ so $p(B|A)$ in [0,1].

p(B|A) indicates the probability that the bacterium occurs B, when the bacterium A is present. If p(B|A) = 1 the bacteria B is always attendant in the presence of bacteria A, consequently p(A|A)=1.
The number of catheter with bacteria species A they need not be equal the number of species B. So, if

$\#(A) \neq \#(B) \rightarrow p(B|A) \neq p(A|B)$.

The probability that the bacterium C occurs in the presence of the bacteria B in the amount of all catheters, that included the bacteria A, is calculated by the probability
of the occurrence of C in B and A ($p(C \mid B \wedge A)$) and the probability that B is in A ($p(B|A)$):

$$p(C| p(B|A)) = p(C \mid B \wedge A)* p(B|A)$$
$$= \#(C\wedge B \wedge A)/\#(B \wedge A)* p(B|A)$$
$$= \#(C\wedge B \wedge A)/\#(B \wedge A)* \#(B \wedge A)/\#(A)$$
$$= \#(C\wedge B \wedge A)/\#(A)$$
$$= p(C \wedge B |A)$$

These calculated values are stored in a csv-table and accessed via MATLAB. MATLAB is a high-level language and interactive environment for numerical computation, visualization and programming. MATLAB is used for data analysis, algorithm development, and the creation of models and applications. To visualize the probabilities of the joint presence of different bacteria species the command 'treelayout' is used. The results show the joint bacteria with A as *Aerococcus* sp., *Proteus* sp., *Pseudomonas* sp., *Enterococcus* sp. and *Escherichia* sp.. Because of p(A|A)=1, all bacteria A

have a probability of 1. The probability of the B (second nodes) and C (third nodes) are described at the y-axis (Figure 5).

2.5.2 Database search

The 16S rRNA gene sequence of *M. odoratimimus* type strain LMG 4029^T (Accession number AJ854059) was uploaded to NCBI BLAST and compared to nucleotide collection. All entries of sequences with similarities of more than 97% were screened for information about the isolation sources or natural habitats of the analyzed bacteria. The NCBI nucleotide collection (http://www.ncbi.nlm.nih.gov /nucleotide/) and the ribosomal database project (http://rdp.cme.msu.edu/) [Cole *et al*., 2009] were screened with the keyword "*Myroides*" and all information about habitats were collected.

2.6 Proteomics

2.6.1 Protein Extraction

One cm of the catheter tip was split in the middle and kept in a urea containing buffer (7 M urea, 2 M thiourea, 50 mM dichlorodiphenyltrichloroethane (DDT), 4% (w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)). Samples were incubated on ice for 5 min, mixed intensively for 30 s and afterwards incubated on ice for 10 min; this step was repeated two times. Sonification was performed for 30 s and repeated five times to dissolve and lyse the cells using a Sonicator (Type UW 2070, Bandelin electronics, Berlin). Subsequently, the catheter was removed and the whole lysate was precipitated with ice-cold acetone (1:7, v/v). After centrifugation (14,000 x g, 40 min) the resulting pellet was dried and dissolved in in 40 µl 1:1 mixture of SDS buffer (1% SDS, 50 mM Tris/HCl, pH 7,5) and urea containing buffer.
Total protein concentrations were determined according to the method of Bradford [Bradford, 1976] using the Coomassie PlusTM Protein Assay

(Thermo Fisher Scientific Inc., USA). The absorbance was measured at 595 nm. The protein concentration was calculated using a bovine serum albumin (BSA) standard (Thermo Fisher Scientific Inc., USA).

2.6.2 One-dimensional SDS-PAGE and tryptic digestion of proteins

The extracted proteins (containing 10 – 30 µg of protein) were separated on a 12% SDS polyacrylamide gel [Righetti, 1990] and stained overnight with colloidal Coomassie Brilliant Blue G-250 as described elsewhere [Neuhoff *et al*., 1988]. Final protein concentrations in the gel were determined by densitometric measurement (Aida Image analyzer v4.15) using a LMW-SDS Marker Kit (GE Healthcare, Germany) as a standard. Eight gel blocks per lane were excised from the gel and digested with trypsin as follows: the excised gel pieces were destained using 50% V/V methanol in 100 mM NH_4HCO_3. Proteins were reduced in 50 mM NH_4HCO_3 containing 10 mM DTT for 30 min at 60 °C and carbamidomethylated/alkylated with 50 mM NH_4HCO_3 containing 50 mM iodoacetamide for 60 min in the dark at room temperature. Subsequently, gel pieces were dehydrated using 100% ACN and allowed to dry. Modified trypsin (sequencing grade, Promega, Germany) was added to a final ratio of 1:10 (trypsin/ sample) in 25 mM NH_4HCO_3 and incubated at 37 °C overnight. Peptides were extracted from the gel by a six step procedure, using acetonitrile, 1% (v/v) formic acid, acetonitrile, 10% (v/v) formic acid and two times acetonitrile. Supernatants-containing peptides were kept, pooled and dried using a Speedvac concentrator (Eppendorf AG). Samples were subsequently resolved in buffer A (5% v/v ACN, 0.1% v/v formic acid) and desalted using ZipTips (C18, Millipore). Finally, peptides were again vacuum-dried and stored at -20 °C.

2.6.3 UPLC-MS/MS analyses and database search

Each peptide mixture was separated by online reverse phase nano ultraperformance liquid chromatography (UPLC) and analyzed by

electrospray tandem mass spectrometry (MS/MS) using an Acquity ultraperformance LC system (Waters Corp.) connected to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). MS/MS samples were dissolved in Buffer A (0.1% formic acid in H_2O) and additionally 2% (v/v) acetonitrile, separated on a C18 reversed phase column via linear gradient using Buffer B (0.1% formic acid in acetonitrile). The raw data from the MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; V2.3.2t). By this step, fractions derived from one sample were pooled. Mascot was set up to search the NCBInr database (11.06.2011, 14,298,189 entries) assuming protein digestion by trypsin. Mascot was searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 10.0 parts per million. Iodoacetamide derivative of cysteine were specified in Mascot as fixed modifications. Oxidation of methionine was specified in Mascot as a variable modification. Decoy searches were performed in a reversed NCBInr database with search parameters identical to those described above.

2.6.4 Protein identification

Scaffold (V3.1.2, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Additionally, the search engine X!Tandem was applied [Craig and Beavis, 2004]. Peptide identifications were accepted if they were established at greater than 95.0% probability as specified by the Peptide Proph*et al*gorithm [Keller *et al*., 2002]. Protein identifications were accepted if they were established at greater than 99.0% probability and contained at least 2 identified unique peptides. Protein probabilities were assigned by the Protein Proph*et al*gorithm [Nesvizhskii *et al*., 2003]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

2.6.5 Further data processing

Taxonomic and functional protein assignments were validated using the Perl script based automated bioinformatics workflow PROPHANE (Proteomics result Pruning & Homology group ANnotation Engine). As a starting point, protein cluster homology was tested by multiple sequence alignment analyses resulting in 40 inconsistent clusters (amino acid identity <50%) which were therefore excluded from further analyses. Subsequently, PROPHANE assigned the remaining protein clusters to phylogenetic and functional groups by searching different databases (i.e. BioPerl, KEGG, KOG/COG, SwissProt) and tested clusters once more for consistency. A detailed description of the workflow is given in the supplement of Schneider and coworkers [Schneider *et al*., 2011].

2.7 Electron microscopic studies

2.7.1 Field emission scanning electron microscopy

Bacterial biofilms were fixed with 2% glutaraldehyde and 5% formaldehyde, washed with TE-buffer (20 mM TRIS, 1 mM EDTA, pH 6,9) before dehydrating in a graded series of acetone (10%, 30%, 50%, 70%, 90%, 100%) on ice for 15 min for each step, critical-point dried with liquid CO_2 (CPD 30, Bal-Tec, Liechtenstein) and covered with a gold film by sputter coating (SCD 500, Bal-Tec, Liechtenstein) before being examined in a field emission scanning electron microscope (Zeiss DSM 982 Gemini, Germany) using the Everhart Thornley SE detector and the inlens detector in a 50:50 ratio at an acceleration voltage of 5 kV.

2.7.2 Infection studies

Infected cells or bacteria were fixed with 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer (100 mM cacodylate, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 90 mM sucrose, pH 6.9). Infection procedure was the same as for immunofluorescence assays. Samples were fixed, washed with PBS and dehydrated in a graded series of acetone (10%, 30%, 50%, 70%, 90%, 100%) and critical point dried with liquid CO_2. Afterwards, samples were covered with a gold film by sputter coating (SCD 500, Bal-Tec, Liechtenstein). Samples were examined in a Zeiss FESEM DSM 982 Gemini (Zeiss, Germany) using the Everhart Thornley SE detector and the inlens detector in a 50:50 ratio at an acceleration voltage of 5 kV [Talay *et al*., 2000].

For transmission electron microscopy samples were fixed with a solution containing 5% formaldehyde and 2% glutaraldyhde in cacodylate buffer for 1 hour on ice, washed with cacodylate buffer and osmificated with 1% aqueous osmium for 1 hour at room temperature. Samples were then dehydrated with a graded series of acetone (10%, 30%, 50%, 70%, 90%, and 100%) for 30 minutes at each step. Dehydration in the 70% acetone step was done with 2% uranyl acetate overnight. Ultrathin sections were cut with a diamond knife, counterstained with uranyl acetate and lead citrate and examined in a TEM910 transmission electron microscope (Carl Zeiss) at an acceleration voltage of 80 kV [von Kockritz-Blickwede *et al*., 2008].

2.8 Infection assays and immunofluorescence analyses

Eukaryotic cells were seeded on 12-mm-diameter glass coverslips placed on the bottom of 24-well tissue culture plates (TPP, Switzerland) and allowed to grow to semiconfluent monolayers. Cells were infected, washed and fixed and samples were processed 3 h after infection. For termination of infection, cells were washed in PBS and fixed with 1% formaldehyde in PBS. To block non-specific antibody binding, cells were incubated with 1% fetal calf serum

(FCS) in PBS for 30 minutes before 1 h incubation with a 1:100 dilution in PBS of the rabbit anti-*Myroides* antiserum. Samples were washed three times with PBS and further incubated for 30 min with a 1:200 dilution of Alexa Fluor 568 goat anti-rabbit IgG antibody (Invitrogen, USA) to stain extracellular bacteria. Then, monolayers were washed three times, permeabilized with 0.1% Triton X-100 in PBS, washed four times and incubated with the rabbit anti-*Myroides* antiserum for another hour. After washing, samples were incubated for 1 h with a 1:200 dilution of Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen, USA) to stain intracellular bacteria. For cytoskeleton staining HEp-2 cells were incubated with Alexa Fluor 568 Phalloidin for 20 min. After the final washing steps, samples were mounted and analysed with a fluorescence microscope (Axiovert 200M, Carl Zeiss). Treatment of non-permeabilized and permeabilized eukaryotic cells with primary and secondary antibodies served as negative controls.

2.9 Fatty acid analyses

Fatty acid analyses were carried out by the Identification Service of the DSMZ, Braunschweig, Germany. Fatty acid methyl esters are obtained from 40 mg cells scraped from Petri dishes by saponification, methylation and extraction using minor modifications of the method of Miller [Miller, 1982] and [Kuykendall *et al*., 1988]. The fatty acid methyl esters mixtures are separated using Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.) which consisted of an Agilent model 6890N gas chromatograph fitted with a 5% phenyl-methyl silicone capillary column (0.2 mm x 25 m), a flame ionization detector, Agilent model 7683A automatic sampler, and a HP-computer with MIDI data base (Hewlett-Packard Co., Palo Alto, California, U.S.A.). Peaks are automatically integrated and fatty acid names and percentages calculated by the MIS Standard Software (Microbial ID). The gas chromatographic parameters are as follows: carrier gas, ultra-high-purity hydrogen; column head pressure 60kPa; injection volume 2 µl; column split ratio, 100:1; septum purge 5 ml/min; column temperature, 170 to 270 °C at 5 °C/min; injection port temperature, 240 °C; and detector

temperature, 300 °C (http://www.dsmz.de/services/services-microorganisms/identification/analysis-of-cellular-fatty-acids.html) [Kämpfer and Kroppenstedt 1996].

2.10 Identification of volatile organic compounds (VOCs) released by *Myroides* spp.

For the analysis of the VOCs released by the microbes the closed-loop stripping analysis was applied [Grob, 1973]. Their chemical nature was determined by GC/MS after standard procedures [Schulz *et al*., 2004]. Bacteria were incubated on LB agar for 24 hours at 37 °C. The volatiles were adsorbed on charcoal (5 mg precision charcoal filter, Chromtech, Idstein, Germany) and eluted with 30 µL of dichloromethane (CH_2Cl_2). The resulting solutions were analyzed by GC/MS spectrometry immediately after elution. GC/MS analyses were carried out on an Agilent GC 7890A system connected to a 5975C mass-selective detector (Agilent) fitted with a HP-5 MS fused-silica capillary column (30 m x 0.25 mm i.d., 0.22 µm film; Agilent). The carrier gas was helium at 1.2 ml min-1 and a splitless injection was carried out. The injection volume was 1 µL, the transfer line 300 °C and the electron energy 70 eV. The GC was programmed to start with 5 min at 50 °C and to increase at a rate of 5 °C min^{-1} to 320 °C. Retention indices were determined from a homologous series of alkanes (C8–C33). Identification of compounds was performed by comparison of mass spectra to those of the NIST Library.

3. Results and Discussion

Most of the published investigations on CAUTI clinically derived material. Often the focus was on severe infections. However, the majority of invasive catheter application results in a biofilm but symptoms for the patient are missing. The question arose if the biofilms on catheters from symptomless patients are the source for severe urinary tract infections. Consequently, 92 catheters were collected with a general practitioner in surgery unit in Kassel, Germany. The corresponding biofilms were systematically investigated for their pathogenic potential. First, their structure was analyzed microscopically. The community structure than was determined using PCR-SSCP and 16S rRNA sequencing. This analysis was accompanied by a metaproteomic approach. Isolates from representative catheter biofilms were investigated for their pathogenic potential. Finally the poorly characterized strain *Myroides* sp A17, also isolated from catheter biofilms was subjected to a throughout phenotypical and genetic analyses.

3.1.1 Urinary tract catheter biofilm analysis using field emission scanning electron microscopy (FESEM)

In order to visualize the microbial communities attached to the inside or outside surfaces of urinary tract catheter surfaces field emission scanning electron microscopy analyses (FESEM) were performed. Typical samples from the surfaces of the devices were analysed and exemplary shown for two catheters (Figure 2). The samples of the catheters were prepared for FESEM as outlined in the method section. Photographs were taken with magnification of 1,000- to 5,000-fold amplification from 2 representative example catheters. Figure 2a shows the FESEM photo of catheter No 5.1 exhibiting the inside of this catheter, while catheter No. 30.2 (Figure 2b) displays the outside biofilm showing again a thick biofilm formation in 5,000 fold magnification with a closer more detailed picture of the multiple bacterial composition within the

biofilm (diameter 0.2 to 0.5 µm). From this catheter the later described *Myroides* sp. strain A17 was isolated and further characterized.

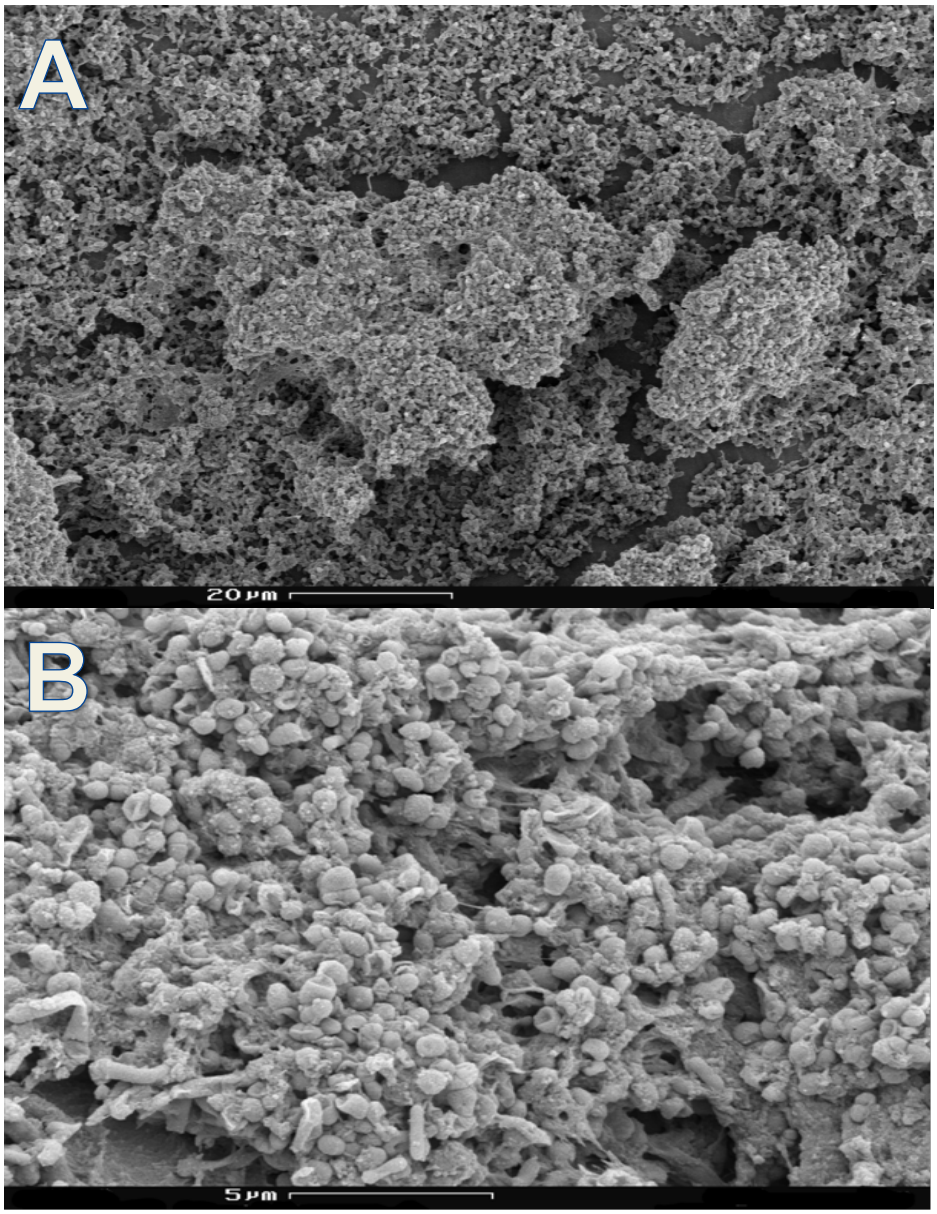

Figure 2 A and B: Field emission scanning electron microscopy analysis of 2 representative urinary tract catheters. The surfaces of the catheters of selected representative evaporators covered with bacteria are shown with 1,000 – 5,000 times enlargement. Panel A, surface of an inner section of catheter No 5.1 showing the thick desiccated ESP matrix; panel B, surface of the outside section of catheter No. 30.1 in the 5,000-fold amplification exhibiting a more detailed picture of the composition of the bacterial biofilm. The FESEM micrographs were taken by M. Rohde, HZI, Germany

The extrapolysaccharide (EPS) matrix seemed to be desiccated (Figure 2A)

There were hardly any differences in the morphology and cell count of the

bacteria. On all tested catheters mostly rod-shapes and filamentous bacteria were detected.

3.1.2 Taxonomic diversity of catheter biofilms detected by SSCP analysis

In this chapter the compositions of the microbial communities of patient-derived catheter biofilms using DNA- as well as protein-based technologies were investigated. While comparison of 16S rRNA gene sequences provided a deep insight into the phylogenetic structure due to the high accessibility in gene libraries, the community proteomic approach allowed a semi-quantitative view on the bacterial community distribution by the use of spectral counting and provides functional information on the complex biofilms.

CAUTI are caused by biofilm formation on the surface and the inner lumen of the catheter. These biofilms can be composed of single or multiple bacterial strains embedded in extracellular polymeric substances (EPS) matrix. To get a deeper insight into the pathogenic-potential of the involved species, it is mandatory to determine the structure of the microbial community of these biofilms. To this end, PCR-based single strand conformation polymorphism (SSCP) with subsequent 16S rRNA gene sequencing was performed.

Figure 3 shows the frequency of detected genera in the catheter biofilms obtained by PCR-SSCP analysis and subsequent 16S rRNA gene sequencing. The complete results of the SSCP analysis are listed in table 2 appendix. *Aerococcus* sp., *Enterococcus* sp., *Proteus* sp., *Morganella* sp., *Staphylococcus* sp., *Corynebacterium* sp., *Stenotrophomonas* sp., *Alcaligenes* sp., *Bacteroides* sp., *Streptococcus* sp., *Escherichia* sp., *Klebsiella* sp., *Actinobaculum* sp., *Prevotella* sp., *Citrobacter* sp. and *Pseudomonas* sp. were found. Other genera were only found once on the catheters. In 84% of all samples a Gram-negative was found together with a Gram-positive species.

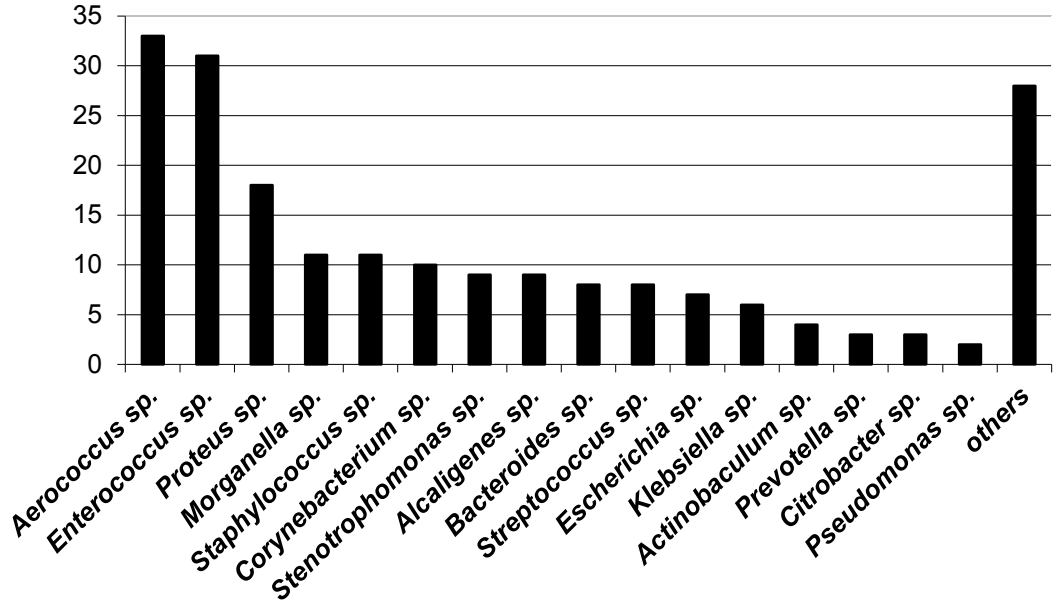

Figure 3: Frequency of species from 44 urinary tract catheter biofilms obtained by PCR-SSCP. The columns indicate the investigated number for each genus. Genera were found from one time to 33 in all 44 catheters.

Species from all these genera have been mentioned in the context of urinary tract infections before [Brook, 2004; Gopalakrishnan *et al*., 1999; Guerra *et al*., 1983; Gupta *et al*., 2012; Jacobsen *et al*., 2008; Liu *et al*., 2004b; Macleod and Stickler, 2007; Olsen *et al*., 2013; Ronald, 2003; Tielen *et al*., 2011]. Interestingly, two of the genera, *Prevotella* sp. and *Bacteroides* sp. are live generally anaerobic. They are common habitants of the human intestine. Since patient's fecal flora is often the contaminating source of the bacteria causing UTI it is not surprisingly that these bacteria were also found in the context of catheter colonization.

3.1.3 Proteome analyses of selected catheter biofilms

To get a deeper insight in the community structure from this obtained result five representative catheters were chosen for further investigation.

Corresponding patient's data of the selected catheters are listed in table 1 appendix. The selected catheter biofilms contained the two most frequent genera found in this study (*Aerococcus* sp. and *Enterococcus* sp.) or the three genera that are most mentioned with the development of complicated CAUTI (*Pseudomonas* sp., *Proteus* sp., and *Escherichia* sp.).

A metaproteomic approach, used before in environmental and human microbiome studies, was chosen to complement the results obtained from the 16S rRNA gene sequencing analyses [Li *et al*., 2011; Schneider *et al*., 2010]. To this end the biofilm was solubilized in buffer and proteins were extracted and analysed by 1D-SDS-PAGE followed by LC-MS/MS as described in the Material & Methods section. Between 150 and 3600 unique bacterial peptides were detected, depending on the biomass recovered from the catheter. Peptides were assigned to corresponding proteins or protein clusters, which were further processed using a metaproteomic analysis software (PROPHANE) defining the smallest common ancestor of the cluster [Schneider *et al*., 2011]. Most of the bacterial peptides were assigned to a certain genus, while only a small number (1 - 4% depending on the biofilm) could not be assigned to a distinct phylogenetic group due to high homologies within the protein clusters ("others").

Obtained results by PCR-SSCP of the selected catheters showed that the communities consisted of Gram-positives as well as Gram-negatives (Figure 4). Members of the Enterobacteriales, Pseudomonadales, Lactobacillales, Xanthomonadales, Actinomycetales and Burkholderiales were detected. The enterobacterium *Proteus* sp. and the Gram-positive *Aerococcus* sp. were found in four out of five catheter biofilms. *Enterococcus* sp. was identified in three catheter biofilms, *Pseudomonas* sp., *Streptococcus* sp and *Escherichia* sp. were each found in two catheter biofilms. The number of different species detected on a single catheter ranged from two (patient 2) to seven (patient 4). Figure 4 shows the results of the metaproteomic studies of community biofilms compared to the SSCP results which were carried out in cooperation with Christian Lassek and Katharina Riedel (Universität Greifswald, Germany).

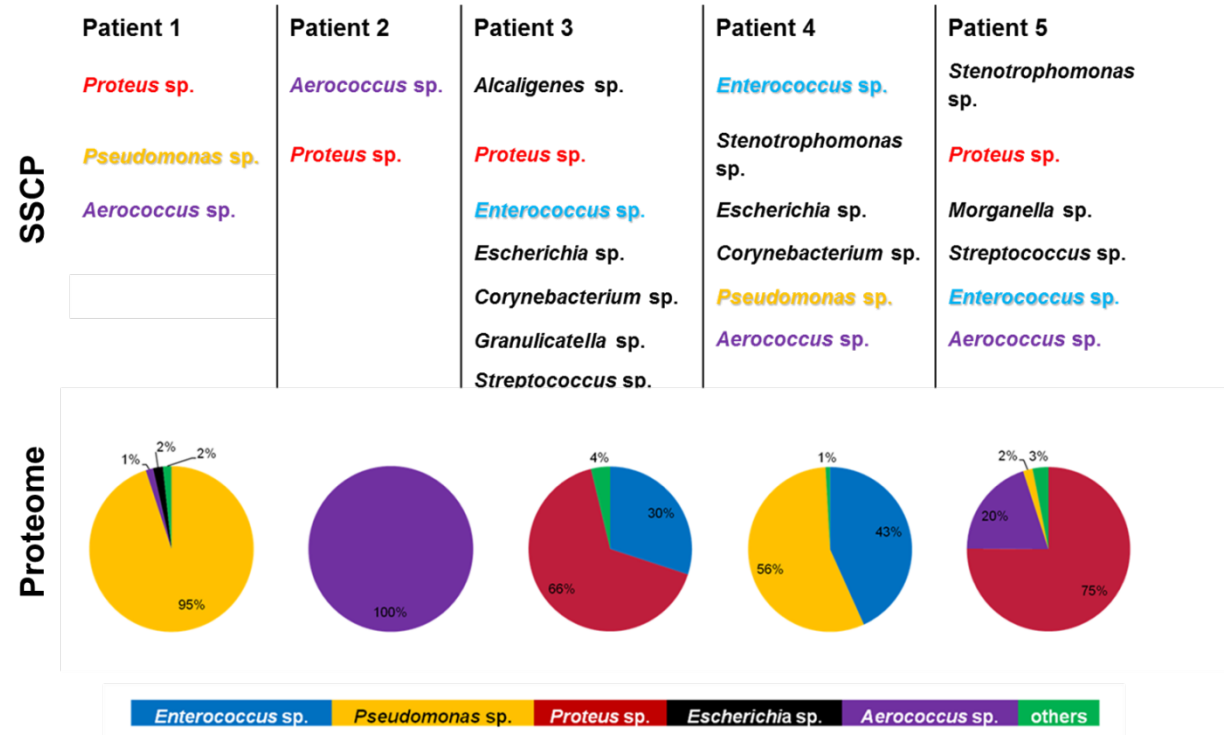

Figure 4: Structure of the microbial biofilms on urinary tract catheters as revealed by PCR-SSCP and the metaproteomic analysis. Quantification is based on spectral counting and subsequent normalization by size of the assigned proteins. A) Results obtained by PCR- SSCP for patient 1 to 5, B) corresponding results obtained by metaproteomic analysis.

Five dominating genera were identified by the metaproteome analyses (Fig. 4): *Enterococcus* sp., *Proteus* sp., *Pseudomonas* sp., *Aerococcus* sp. and *Escherichia* sp..

The catheter biofilm of patient 1 was dominated by *Pseudomonas* sp. (95% of assigned proteins). Moreover, *Aerococcus* sp. (1%) and *Escherichia* sp. (2%) were found.

In the catheter biofilm of patient 2 only proteins assigned to *Aerococcus* sp. were found.

Proteins that were extracted from the catheter biofilm of patient 3 revealed the presence of *Proteus* sp. (66% of assigned proteins) and *Enterococcus* sp. (30%).

In the catheter biofilm of patient 4 mainly *Enterococcus* sp. (56% of assigned proteins) and *Pseudomonas* sp (43%) proteins were found.

The catheter biofilm of patient 5 contained proteins from *Proteus* sp. (75% of assigned proteins), *Aerococcus* sp. (20%) and *Enterococcus* sp. (2%), respectively.

Subjecting patient 1, these results confirmed the results obtained from PCR-SSCP as the three species found were *Pseudomonas* sp. and, *Aerococcus* sp. (Fig. 4). As the abundance of *Escherichia* sp. is very low this information may have got lost in the amplification step of the PCR-SSCP. Both methods revealed the presence of *Aerococcus* sp. in the catheter biofilm of patient 2 whereas the proteome revealed only the abundance of *Aerococcus* sp. The proteome of patient 4, validated SSCP results with regard to the presence of *Pseudomonas* sp. and *Enterococcus* sp.. Both methods are in good agreement with the finding of *Enterococcus* sp. and *Proteus* sp. in the catheter biofilm removed of patient 3 (Figure 4). It is expected that abundance of rarely or uniquely found species in the SSCP analyses is under the detection limit of the proteomic approach. In the catheter biofilm of patient 5, both methods revealed the presence of *Proteus* sp., *Enterococcus* sp. and *Aerococcus* sp.. SSCP and metaproteomics showed that *Aerococcus* sp. and *Proteus* sp. are the most frequent genera of organisms found in catheter biofilms (Figure 4).

One of the most frequent bacteria mentioned in context with CAUTI or UTI is *P. aeruginosa*. It constitutes the third most isolated bacterium [Manfredi *et al*., 2000]. The Gammaproteobacterium produces various virulence factors and is well adapted to the urinary tract for example by producing alginate embedded biofilms [Mittal *et al*., 2009; Tielen *et al*., 2010]. Similarly, the both Enterobacteriaceae *Proteus mirabilis* and *Escherichia coli* are well studied [for review see:Jacobsen *et al*., 2008]. Furthermore, the two enterococcal species, *E. faecalis* and *E. faecium* are recurrent isolated from urinary tract catheters. The genus *Aerococcus* belongs to the Gram-positive Lactobacillales. Some *Aerococcus* species have been implicated previously in the context of urinary tract infections. *Aerococcus urinae* was isolated from urine samples from patients with urinary tract infections and described as a new *Aerococcus* species in 1992 [Aguirre and Collins, 1992]. Furthermore,

Aerococcus viridans has been described as the causative agent of UTI in a pediatric patient [Leite *et al*., 2010] and a survey from a Danish group revealed that approximately 0.8% of all urinary tract specimens were positive for *Aerococcus*-like organisms [Christensen *et al*., 1989].

In total, 13 different genera on five catheter biofilms by PCR-SSCP and 5 out of these 13 genera by metaproteomics approach were detected. SSCP is a highly sensitive and well established method to elucidate the microbial community, but it is not quantitative. The modern methods of metaproteomic analysis are less sensitive compared to the SSCP method, but complement the SSCP results by providing quantitative information about the microbial community and highlights abundant genera. Interestingly, four out of five of the bacterial biofilms on urinary tract catheters investigated in this study were composed of multiple genera. However, if a biofilm was co-dominated by two genera (as in patients 3, 4 and 5), a Gram-negative and a Gram-positive organism were found. In none of the specimens, two species with the same Gram type were co-dominant.

Microbial biofilms of five catheter-associated urinary tract biofilms were analyzed by DNA-based SSCP and a metaproteomic approach. 16S rRNA gene sequencing indicated a high diversity in the biofilms with 2-7 different bacterial species per biofilm. The complementary metaproteomic approach demonstrated a comparably high diversity, but also reveals that each biofilm was dominated by one or two genera, namely, *Pseudomonas* sp., *Proteus* sp., *Enterococcus* sp. and *Aerococcus* sp.. When a biofilm was co-dominated, a Gram-negative and a Gram-positive organism were found; never two genera with the same Gram character were co-dominant.

3.1.4 Statistic analyses of catheter biofilm communities revealed the obligate presence of at least one Gram- positive or Gram negative bacterium

Interestingly, every bacterial community from the catheter biofilms was always composed of Gram-positive as well as Gram-negative bacteria. The 16S rRNA genes sequences that were related to *Enterococcus* sp. were found two times in combination with *Stenotrophomonas* sp, *Escherichia* sp. and *Proteus* sp., respectively.

The frequency of a joint presence of two bacteria species was determined for the SSCP data sets (table 2 appendix) using the open source programming language R package as described in the material and methods section. Figure 5A-E shows the resulting trees. There, the probabilities that coming from one species one of the others is present is drawn. When *Aerococcus* sp. was present in the catheters, the likelihood for *Enterococcus* sp. was over 60%, that for *Proteus* sp. about 50%. On the other hand, *Enterococcus* sp. was also mostly found with *Aerococcus* sp., followed by *Proteus* sp. and *Enterococcus* sp.. If *Pseudomonas* sp. was present in the community with *Proteus* always also *Aerococcus* sp. and *Enterococcus* sp. were present.

The most interesting finding was that when *Pseudomonas* sp. was found it was always (100%) coming along with *Enterococcus* sp. and *Aerococcus* sp.. Thus, if one of the other species was found (both 50%) they were also linked with *Enterococcus* sp. and *Aerococcus* sp.. Similarly, *Escherichia* sp. was accompanied with *Aerococcus* sp. and *Enterococcus* sp. in more than 80%.

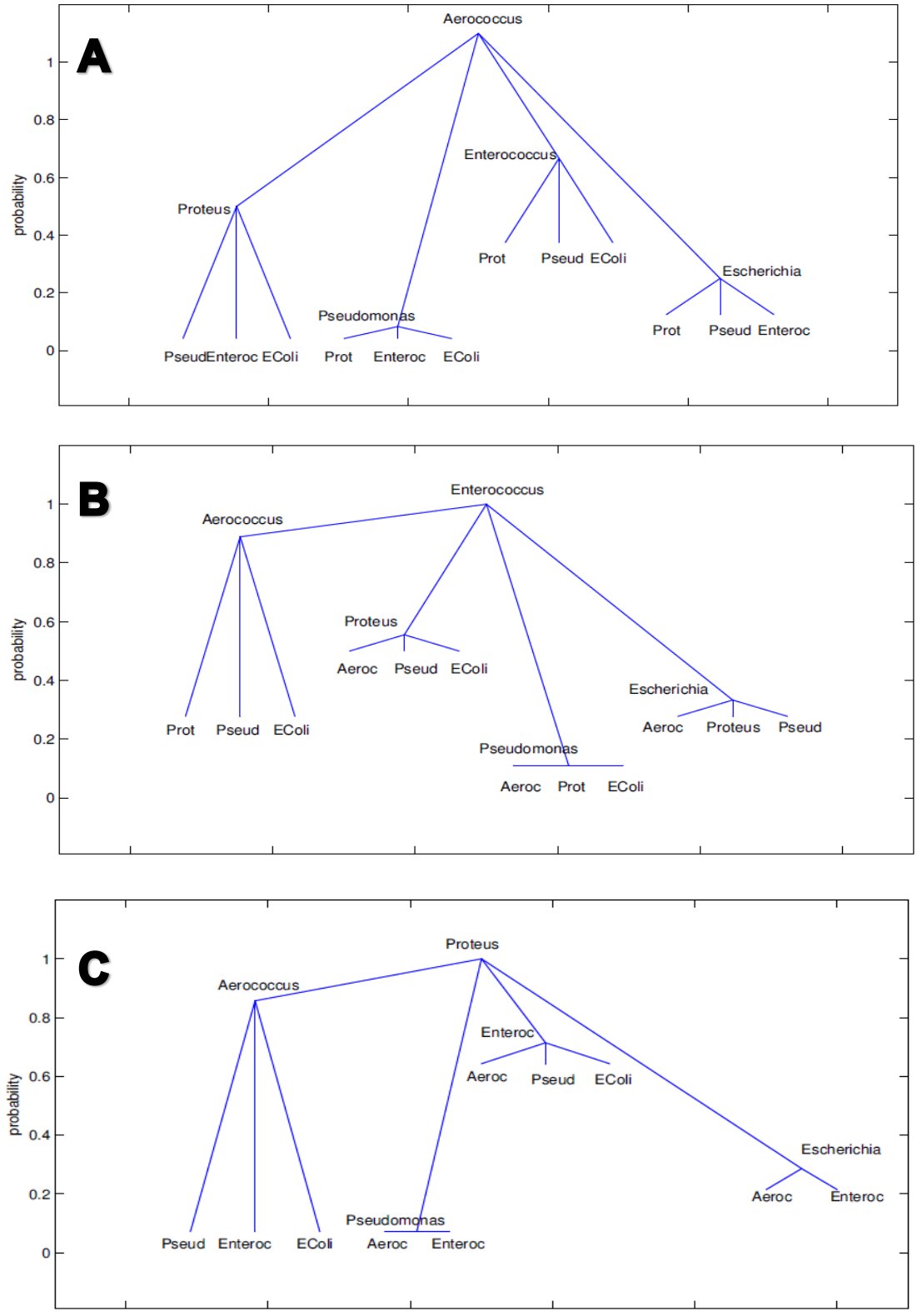

Figure 5 Joint venture trees of A) *Aerococcus* sp., B) *Enterococcus* sp., C) *Proteus* sp., D) *Pseudomonas* sp. and E) *Escherichia* sp.. The distances indicate the probability that bacterium A occurs if bacterium B is present. Detailed information in chapter 2.5.1

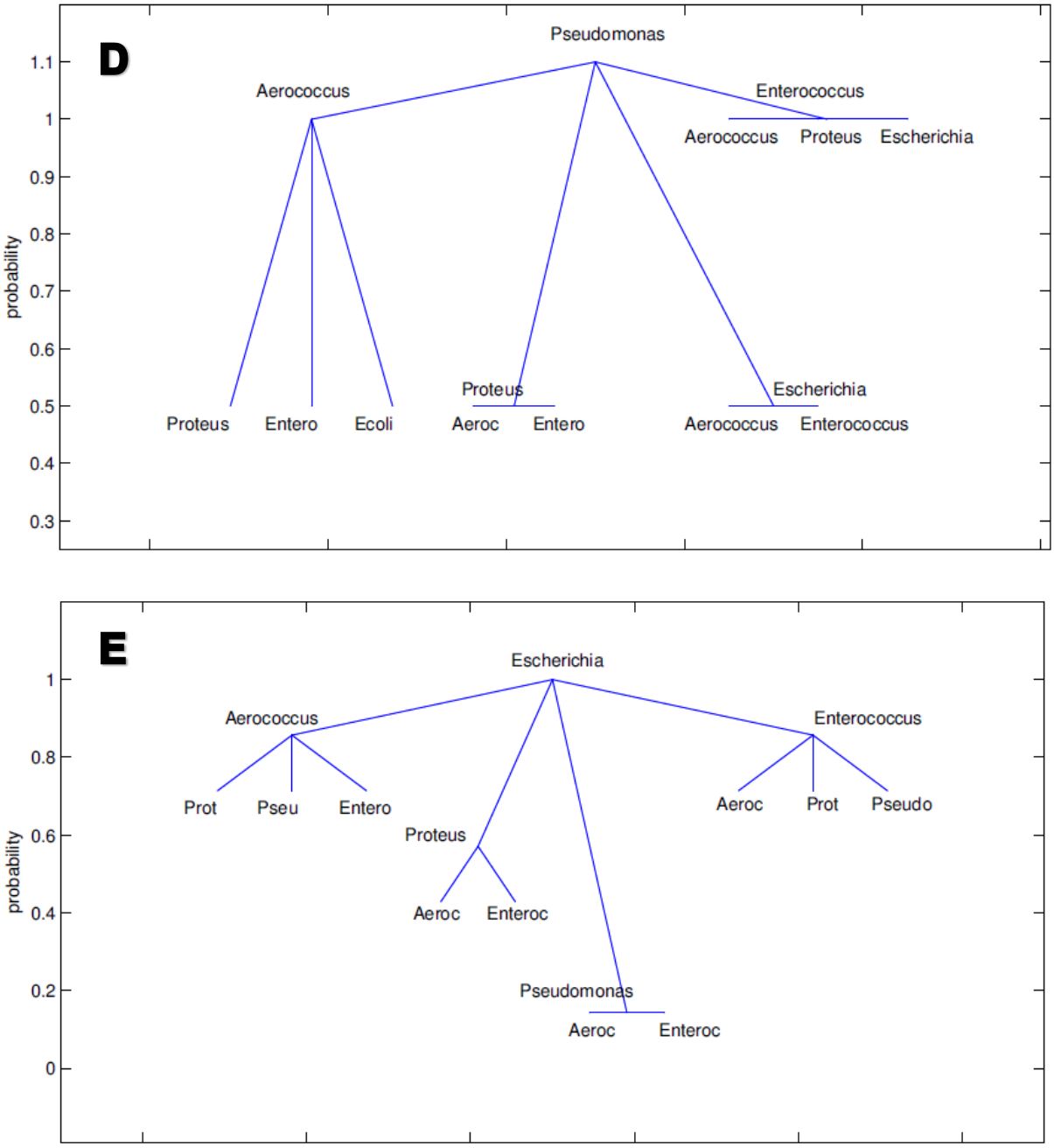

Figure 5, continued

Remarkable is the finding that, if Gram-negative genera were found in urinary tract catheter biofilms, the probability of an accompanying Gram-positive was extremely high. 84% of the communities obtained via PCR-SSCP were composed of Gram-negatives as well as Gram-positives. This leads to the conclusion that communities with mixed Gram characteristics are the most

stable and successful. Of course, this hypothesis needs to be validated by functional metaproteomic analyses.

3.2 Isolation, identification and characterization of bacteria from catheter biofilms

Microbial biofilms on catheters are a major cause of catheter blockage which usually results in the cost intensive and the risky exchange of the device. Often catheterized patients do not show any obvious symptoms despite that fact that an immune response is triggered by the invasive catheter application process. More importantly, these biofilms represent a potential reservoir for urinary tract infections like severe CAUTI. These CAUTI constitute an important problem for many physicians in clinics and practices. In contrast to its medical impact only few systematic investigations at the multibacterial level combining identification and phenotypic characterization of catheter colonising bacteria can be found in the literature. Most data focus on hospital acquired CAUTI of urine samples. Less attention has been paid to the identity and properties of catheter biofilm bacteria from patients without obvious symptoms. The question for their potential to cause severe CAUTI was investigated in this study. Consequently, bacterial strains from catheter biofilms from urological practice were isolated to test them for their biofilm forming potential, their ability to utilize urea, to produce DNase and proteases, to perform hemolysis, their motility and finally their antibiotic resistance profiles.

3.2.1 Nine different isolates were isolated from catheter biofilms that are potential urinary tract pathogens

A total of 92 catheters from patients without symptoms of infection but biofilm on their devices were subjected to strain isolation and identification. A total of 15 different strains were isolated and identified. Species were isolated using

LB-agar, MacConkey, TSA, Corynebacterium agar and CLED, respectively. They were purified to monoculture by transferring single colonies to fresh appropriate medium for three consecutive cycles. As all strains were able to grow on LB-agar; this medium was used for further experiments. To identify the isolated strains 16S rRNA gene sequencing was performed. Bioinformatic interpretation of obtained DNA sequences was carried out using the corresponding tools at NCBI [Altschul *et al*., 1990]. The 16S rRNA gene sequences identified the nine isolates as *P. mirabilis*, *M. a morganii*, *P. aeruginosa*, *A. faecalis, E. faecalis*, *S. maltophilia*, *Myroides* sp*, S. aureus* and *Enterobacter cloacae,* respectively. Out of all isolates nine representative strains were selected for further analysis. Table 2 summarizes the results of the strain isolation and the corresponding patient's data. Strains were isolated from catheters of male patients except for *Myroides* sp. which was an isolated from a 91 year old female. The age of the patients ranged from 75 to 92 years.

The enterobacterium *P. mirabilis* is commensal of the human colon and an opportunistic pathogen causing classical nosocomial infections [Coker *et al*., 2000]. In the environment the bacterium lives as organotrophic saprophyte. It is known to cause wound infections, pneumonia, sepsis and urinary tract infections [Coker *et al*., 2000; Toth and Emody, 2000]. *P. mirabilis* is the third most common cause of complicated UTI (12%) [Jacobsen *et al*., 2008]. *M. morganii* can be found in the environment and the intestinal tract of mammals. However, it also causes symptoms like sepsis, ecthyma, endophthalmitis, chorioamnionitis and most commonly urinary tract, skin and soft tissue infections, as well as meningitis [Kim *et al*., 2007; Macleod and Stickler, 2007]. *P. aeruginosa* is a well characterized opportunistic pathogen involved in multiple infections of the lung, eye and of burn wounds [Schobert and Jahn, 2010]. Moreover, *P. aeruginosa* infections of the lung of patients suffering from cystic fibrosis often cause detrimental outcomes. Recently, *P. aeruginosa* strains from urinary tract infections were described and characterized [Tielen *et al*., 2011].

Table 2: Identified bacteria isolated from Foley´s catheters replaced from 8 different patients.

Bacteria data			**Patients data**	
Strain-no.	**Species**	**Medium for isolation**	**Sex**	**Year of birth**
A1	*Proteus mirabilis*	MacConkey	m	1936
A2	*Morganella morganii*	LB	m	1924
A6	*Pseudomonas aeruginosa*	LB	m[a]	1920[a]
A7	*Alcaligenes faecalis*	MacConkey	m	1926
A8	*Enterococcus faecalis*	LB	m[a]	1920[a]
A14	*Stenotrophomonas maltophilia*	LB	m	1923
A17	*Myroides* sp.	TSA	f	1936
A22	*Staphylococcus aureus*	Corynebacterium agar	f	1922
A24	*Enterobacter cloacae*	CLED	m	1946

[a]same patient; m= male, f= female

A. faecalis is ubiquitously found in the nature and rarely an opportunistic pathogen [Kahveci *et al*., 2011]. However, cases of urinary tract infections associated with *A. faecalis* have been reported [Guerra *et al*., 1983]. The Gram-positive *E. faecalis* belongs to the order of Lactobacillales. It is commonly found in the intestine of human and animals and causes various nosocomial infections of the urogenital tract. *Myroides* sp. belongs to the Flavobacteriales. Members of the genus (formerly *Flavobacterium*) are wide spread in the environment and are usually regarded as low-grade opportunistic pathogens. However, the involvements in endocarditis, ventriculitis, sepsis, pneumonia, soft skin cutaneous and urinary tract

infections have been reported [Benedetti *et al*., 2011]. *S. maltophilia* is known to cause hospital-acquired infections and it has been implicated in catheter-related bacteremia and septicemia, urinary and respiratory tract infections, mastoiditis, conjunctivitis and endocarditis [de Oliveira-Garcia *et al*., 2003; Gopalakrishnan *et al*., 1999; Schoch and Cunha, 1987]. Both species, *S. aureus* and *E. cloacae* were mentioned in the context of community acquired infections before [Liu *et al*., 2004a]

S. aureus is a well characterized nosocomial pathogen. It´s relevance is increasing rapidly due to broad range antibiotic resistances, especially for Methicillin-resistant *S. aureus* (MRSA) strains. Severe infections include necrotizing pneumonia [Kreienbuehl *et al*., 2011], pyomyositis [Burdette *et al*., 2012], sepsis [Bassetti *et al*.], osteomyelitis [Kechrid *et al*., 2011] and necrotizing fasciitis [Changchien *et al*., 2011; Watkins *et al*., 2012].

The enterobacterium *Enterobacter cloacae* has been mentioned in the context of urinary tract infections, pyomyositis and blood stream infections, for example [Gousseff *et al*., 2013; Hilty *et al*., 2013; Qian *et al*., 2012].

3.2.2 *E. coli* is missing in biofilm from catheters from patients without symptoms

Notably, of the previous in the context of CAUTI and urinary tract infections isolated abundant bacteria, only *E. coli* [Bhatia *et al*., 2010] was not found on the catheters from the here examined asymptomatic patients. Nevertheless, this observation remains remarkable and might point towards significant difference in the biofilm composition between catheters from asymptomatic patients and those with CAUTI. Obviously, in some cases the occurrence of CAUTI with hazardous symptoms is accompanied with the dominant presence of *E. coli* strains. This represents characteristics for the shift from asymptomatic catheter colonisation to CAUTI.

3.2.3 Biofilm formation of the strains isolated from catheters and the influence of iron limitation

In order to determine the biofilm forming potential of the urinary tract isolates 96 well assays and subsequent CFU determination were performed. The results revealed that all species were capable of biofilm formation. Figure 6 shows the biofilm formation potential in LB medium (black columns), AUM (dark grey columns) and the determination of the CFU (light gray columns) of all isolated strains. The strongest biofilm formation potential was observed for *P. aeruginosa* (LB) and *A. faecalis* (AUM). *S. maltophilia* also formed thick biofilms in both media. The both Gram-positive bacteria *E. faecalis* and *S. aureus* showed the least biofilm formation in AUM compared to the other isolates.

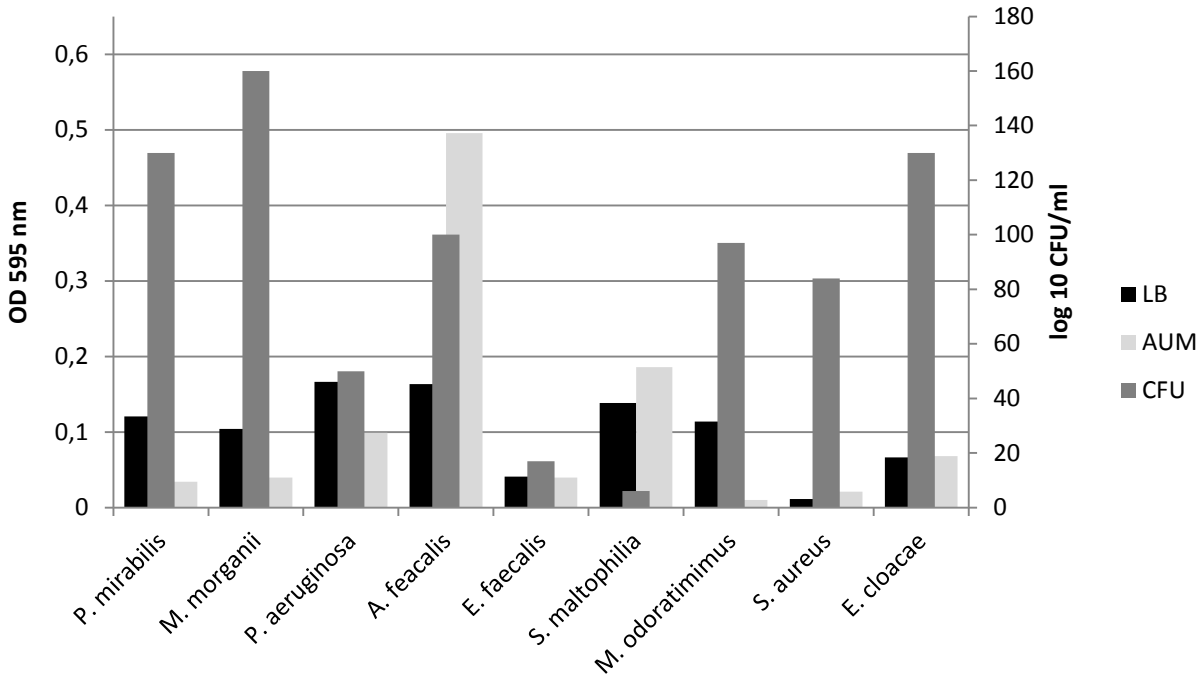

Figure 6: Biofilm formation potential of *P. mirabilis*, *M. morganii*, *P. aeruginosa*, *A. faecalis*, *E. faecalis, S. maltophilia*, *M. odoratimimus, S. aureus* and *E. cloacae* isolated from catheter biofilms. The columns in black and dark gray show the biofilm forming potential in LB and AUM, respectively. The columns in light gray indicate the corresponding numbers of colony forming units in LB. Biofilms were grown for 24 h in LB or AUM using microtiter tissue plates. Crystal violet staining of the biofilms was measured at OD 595 nm. For CFU determination suspended cells from the biofilm were plated onto LB agar.

This can be explained by the fact that both strains did not grow well in the iron limiting AUM. However, growth of all other strains was not affected by the iron limiting conditions in the AUM medium. In order to validate the true biofilm formation of the bacteria and to exclude the artificial staining of secreted material that binds to the wells of the microtiter plate additional determination of CFUs were performed. Surprisingly, the results showed that the thickness of the biofilm does not correlate with the number of the bacteria that were enclosed in the biofilm. For example, *P. aeruginosa* which formed a very thick biofilm in both media showed a CFU of $5 * 10^8$ which was less than 50% of the CFU of *M. morganii*. On the other hand, S*. aureus* showed a high number of CFU ($8.4 * 10^8$) but built in contrast a very thin biofilm.*S. maltophilia* also formed a strong biofilm, but only little is known about biofilm formation by this organism. Adherence to epithelial cells and abiotic surfaces has been described before [de Oliveira-Garcia *et al*., 2003]. Similarly, *E. faecalis* is capable of biofilm formation and infection of urethral stents [Minardi *et al*., 2011; van der Waal *et al*., 2011]. *A. faecalis* biofilms were found on nitrate-rich industrial effluents and in a fuel consuming consortium [Jadhav *et al*., 2005; Pepi *et al*., 2003]. *M. odoratimimus* and *M. odoratus* biofilms were found on ship´s hull and on fish [Inbakandan *et al*., 2010; Jacobs and Chenia, 2009]. *M. morganii* was less abundant identified in urinary tract infections [Macleod and Stickler, 2007]. In contrast, *P. aeruginosa* and *P. mirabilis* biofilms in the context of urinary tract infections are well characterized [Jacobsen and Shirtliff, 2011; Kazmierska *et al*., 2010; Mittal *et al*., 2009]. Furthermore, the ability of *S. aureus* to form biofilms is an important virulence mechanism that complicates infections [Watkins *et al*., 2012]. Moreover, *E. cloacae* is known for its ability to form biofilms. Some strains were found to be adhesive to human intestinal cell lines [dos Reis Ponce *et al*., 2012; Livrelli *et al*., 1996]. An involvement of curli fimbriae in the biofilm formation of *E. cloacae* was described in the literature before [Kim *et al*., 2012b].

In contrast, this is the first description of biofilm formation of *S. maltophilia*, *A. faecalis* and *M. odoratimimus* in the context of catheter colonisation.

The inconsistency of biofilm thickness observed by the staining procedure and the determination of real cell numbers using the CFU method can be explained by the differences in the secretion of EPS by the various strains. The secretion of EPS and thereby the thickness of biofilms is affected by the cell to cell signaling [Davies *et al*., 1998]. However, extensive secretion of more substances can lead to a thicker biofilm even though the number of cells is small.

3.2.4 Urease activity of the biofilm forming catheter isolates

Urea is the main nitrogen containing compound in urine (about 9 g L^{-1} [Putnam, 1971]) Hydrolysis leads to the formation of ammonium and carbon dioxide. This process changes the acidic pH of the urine to alkaline conditions which result in the precipitation of dissolved salts on the catheter surface blocking the catheter flow [Elliot *et al*., 1959].

Urease production has also been implicated with bladder and kidney stone formation [Stamm, 1991]. In order to test for the ability of the isolates to hydrolyze urea 96 well microtiter plate assays were performed. All species were able to hydrolyze urea. *P. mirabilis* was found the strongest urease producer. *P. mirabilis* urease activity value was set 100% and all other values were related to that (Figure 7). *A. faecalis* and *E. faecalis* revealed more than 80% of the urea consumption, *S. aureus and S. maltophilia ranged* around 50%, while *M. morganii,* and *P. aeruginosa* showed less than 40%. The encrustation of the catheter is often referred to *Proteus* sp. [Kunin, 1989]. *P. mirabilis* is characterized by a strong urease activity which is usually employed for the classical identification of the organism [Henriksen, 1950]. *M. morganii* urease is a well biochemically characterized enzyme [Hu *et al*., 1990]. Like *Yersinia enterocolitica* urease, it is activated at low pH and enhances the survival of the bacteria in acidic conditions [Young *et al*., 1996]. Although *P. aeruginosa* has a urease it fails to cause crystal deposition in the biofilm [Broomfield *et al*., 2009]. The urease activity of *S. aureus* was found increased under biofilm forming conditions like such found on catheters [Beenken *et al*., 2004]. *E. cloacae* urease was found to only slightly raise the

pH of the urine and failed to cause crystal deposition in the biofilm [Broomfield *et al*., 2009].This is the first record of urease activity of *A. faecalis*, *E. faecalis* and *M. odoratimimus* in the context of catheter colonisation

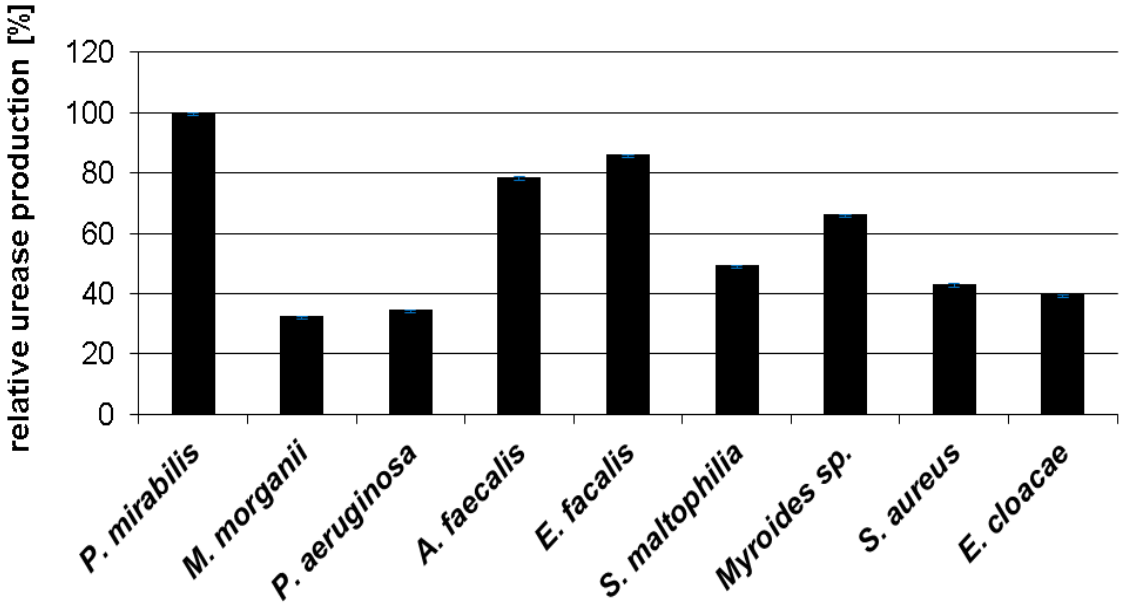

Figure 7: Urease production of *P. mirabilis*, *M. morganii*, *P. aeruginosa*, *A. faecalis*, *E. faecalis*, *S. maltophilia*, *M. odoratimimus, S. aureus* and *K. pneumoniae* isolated from CAUTI associated biofilms. The urease production of *P. mirabilis* was set 100% and all other values were related to that. The columns indicate the relative urease production of the strains. Urease hydrolysis was monitored in microtiter tissue plates. Cells were grown in Urea Broth and the change of the pH indicator (phenol red) after 24 h was measured at 595 nm.

All urinary tract isolates were also able to hydrolyze urea and thereby enhance the risk for complicated CAUTI. Crystalline biofilms can cause trauma to the bladder and urethral epithelia [Stickler, 2008]. Consequently, urease inhibitors may decrease development of complicated urinary tract infections. Interestingly, clinical trials revealed reduced numbers of viable *Helicobacter pylori* cells by urease inhibition for treatment of chronic gastritis [Stoschus *et al*., 1996].

3.2.5 Hemolysis, DNase, protease activity, and motility of the catheter isolates

Hemolysis is the breakdown of erythrocytes with the release of hemoglobin and other cell constituents usually caused by hemolysins. Most hemolysins are proteins, but others like rhamnolipids and biosurfactants have been described [Ortiz *et al*., 2010; Ortiz *et al*., 2006; Sanchez *et al*., 2010; Sanchez *et al*., 2006]. Many bacteria utilize the released heme as iron source [Letoffe *et al*., 1994].

DNases degrade DNA to their deoxyribonucleotides building blocks which are taken up and utilized by microorganisms. Moreover, extracellular DNA fragments can contribute to the extracellular matrix of biofilms. DNase activity is typical for many bacterial pathogens [Elsner *et al*., 2000; Sumby *et al*., 2005].

Protease can contribute to the bacterial pathogenesis basically by two different routes: in Gram-positive bacteria the Clp proteases control the expression and production of several virulence factors; in Gram-negative bacteria proteases play an important role by affecting the bacterial envelope [Ingmer and Brondsted, 2009]. Bacterial motility is well studied in the context of UTI for *E. coli*. It can contribute to the virulence of bacteria by ascending upper sites of the urinary tract or escape the immune response of the host [Lane *et al*., 2005]. It can be mediated by either swimming, swarming or twitching motility.

Tests for hemolysis, DNA degradation and proteases were performed on agar plates containing blood, nucleic acid and skim milk, respectively. Tests for motility were performed as described in the material and methods section. The results for hemolysis, DNase and protease and motility activity are shown in Table 3. Hemolysis was detected for *P. aeruginosa, S. maltophilia* and *Myroides* sp.. DNA was hydrolyzed by all strains except of *E. faecalis, S. aureus* and *E. cloacae*. All strains except for *A. faecalis* and *E. faecalis* produced proteolytic enzymes. Motility was only shown for *M. morganii, P. aeruginosa, A. faecalis* and *E. cloacae*. *P. mirabilis* was not able to grow on

the motility test medium but showed strong swarming on LB medium. Interestingly, hemolysis has been described for uropathogenic *P. mirabilis* isolates before [Sosa and Zunino, 2009; Toth and Emody, 2000]. Similarly, strong hemolytic activity was documented for various *M. morganii* [Goluszko *et al*., 1988] and *E. faecalis* strains [Lindenstrauss *et al*., 2011]. Nevertheless, the analyses failed to detect significant hemolysis activities by the catheter associated isolates. This must represent a specific adaption to the individual urogenital tract since hemolysis by the organisms was mainly found during sepsis, on wounds and by environmental isolates [De Vuyst *et al*., 2003; Huycke *et al*., 1991; Kim *et al*., 2007]. This kind of bacterial adaptation was also found in *E. coli* isolates from patients with asymptomatic bacteruria [Dobrindt, 2010; Salvador *et al*., 2012]. Moreover, there were reports of hemolysin free *M. morganii* strains [Goluszko *et al*., 1988]. In agreement with these findings hemolysis has been reported for *S. maltophilia* [Figueiredo *et al*., 2006] and several times for *P. aeruginosa* [Puzova *et al*., 1994].

Table 3: Table 3: Hemolysis, protease, DNase activity and motility of the isolated strains. Tests were carried out on Columbia Agar with sheep blood, DNase test agar plates with methyl green and protease test agar containing skim milk powder. Motility was tested on M9 medium with decreased agar concentrations [O'Toole and Kolter, 1998]

Species	Hemolysis	DNase	Protease	Swimming	Swarming	Twitching
P. mirabilis	-	+	+	nG	nG	-
M. morganii	-	+	+	+	+	+
P. aeruginosa	+	+	+	+	+	+
A. faecalis	-	+	-	-	+	-
E. faecalis	-	-	-	nG	-	-
S. maltophilia	+	+	+	-	-	-
Myroides sp.	+	+	+	nG	nG	-
S. aureus	-	-	+	-	-	-
E. cloacae	-	-	+	+	+	-

+ = positive, - = negative, nG = no growth

Similarly, no documentation of hemolysis activity by *A. faecalis* was available in the literature. Interestingly, hemolysis has not been demonstrated for *Myroides* strains before. Therefore, this is the first description on a hemolytic *Myroides* isolate. However, since only 3 out of 9 isolates revealed hemolytic activity, this parameter might not significantly contribute to catheter colonisation. Taking the urinary tract conditions into account this observation makes sense. The DNase profile is in a good agreement with previous findings including *E. faecalis* and *E. cloacae* strains without DNase activity [Elsner *et al*., 2000]. In contrast there are reports of *S. aureus* DNase-positive strains. Interestingly, it was reported that secretion of nucleases and thereby a low concentration of extracellular DNA limits biofilm formation [Beenken *et al*., 2004; Mann *et al*., 2009; Tsang *et al*., 2008].

P. aeruginosa is well studied opportunistic pathogen that releases several proteases. These enzymes contribute to the virulence of the bacterium by affecting the biofilm formation or cleaving immunoglobulins [Döring *et al*., 1981; Tielen *et al*., 2010]. Moreover, it was shown that low level protease producing strain of *P. aeruginosa* were less virulent in mice [Snell *et al*., 1978]. Some strains of *P. mirabilis* can produce a protease that is able to cleave IgA which is secreted among others in the urogenital mucus [Senior *et al*., 1987]. The same authors mentioned in another publication that strains of *M. morganii* lacking this enzyme [Senior *et al*., 1988]. There are no reports of protease production of *Myroides* sp. strains in the context of urinary tract infections. However, there is a report of a metalloprotease with elastinolytic activity in a strain of *M. profundi* [Chen *et al*., 2009]. *S. maltophilia* has at least three different proteases that contribute to the virulence of the bacterium in a *Galleria mellonell*a larvae model [Nicoletti *et al*., 2011]. The detection of protease activity for the *E. faecalis* and *A. faecalis* isolates failed. However, there were reports of protease producing strains of *E. faecalis* and *A. faecalis* from others [Sifri *et al*., 2002; Thangam and Rajkumar, 2002]. Interestingly, most isolated strains processed the typical virulent phenotype of CAUTI-causing bacteria.

Motility of bacteria contributes to virulence by the fact that bacteria are able to enter the urinary tract and there ascend *via* the urethra to make new infections sites accessible [Herrmann and Burman, 1985; Stamney, 1980]. Motility was found for all Enterobacteriaceae, *P. aeruginosa* and *A. faecalis*. However, some strains were not able to grow on the tested minimal medium. Although the role of motility has been discussed for uropathogens before, the role for biofilm associated isolates still needs to be determined.

3.2.6 Antibiotic resistance

Multiple antibiotic resistances of microbial biofilms significantly contribute to the development of complicated CAUTI [Kurtaran *et al*., 2010; Milan and Ivan, 2009]. In order to determine the resistance profiles of the urinary tract catheter isolates antibiotics from six different classes usually employed for the chemical treatment of bacterial infections were tested. Table 4 shows the results of the antibiotic resistance assays. Areas highlighted in gray indicate sensitivity of the tested isolate to the antibiotic. Interestingly, none of the tested substances limited the growth all of the urinary tract isolates. The most effective antibiotic was gentamicin that inhibited the growth of all strains except of *M. odoratimimus* (MIC = 8 mg/L). Ciprofloxacin, tobramycin and levofloxacin each inhibited each the growth of 2 strains. The antibiotics ampicillin, cotrimoxacole and cefixime had no effects on the isolated strains even at high concentrations. All strains except of *P. mirabilis* showed high MICs (>75 mg/L) against cefixime (cut off value 1 mg/L). Nitrofurantoin showed no inhibitory effects on the growth of the isolates up to high concentrations of 260 mg/L. Some strains were weakly sensitive to the solvent which contained dimethylformamide. The substance was reported to inhibit bacterial growth during quantitative susceptibility tests [Barry and Lasner, 1976]. The *A. faecalis, P. aeruginosa* and *S. maltophilia* strains were not affected by all antibiotics except for gentamicin. *P. aeruginosa* and *P. mirabilis* urinary tract isolates are well studied for antibiotic resistance

[Obritsch *et al*., 2005; Saito *et al*., 2007]. In good agreement with this results ciprofloxacin, gentamicin and tobramycin resistances have been reported for *P. aeruginosa* urinary tract isolates [Cernohorska and Slavikova, 2009; Rodriguez *et al*., 2006]. In contrast to previous investigations, the *P. mirabilis* isolate was sensitive to ciprofloxacin treatment. In agreement with these findings, previous studies showed that *S. maltophilia* strains are highly resistant to different classes of antibiotics, such as aminoglycosides and ß-lactams [Morrison *et al*., 1986]. Additionally, the *S. maltophilia* isolate was resistant to cephalosporin and fluorochinolon antibiotics. *A. faecalis* strains were found to produce an extended-spectrum ß -lactamase [Pereira *et al*., 2000] which agrees with the results obtained in this study. The antibiotic resistance profile of *A. faecalis* observed in this investigation differed only slightly to a previous report [Bizet *et al*., 1993].

Table 4: Determined minimal inhibitory concentrations (MIC) of the isolated strains for the antibiotics ampicillin (Amp), cefixime (Cef), ciprofloxacin (Cip), cotrimoxazole (Cot), nitrofurantoin (Nit), tobramycin (Tob), levofloxacin (Lev) and gentamicin (Gen). Areas of the table highlighted in gray indicate that the bacterium is sensitive to the antibiotic treatment while areas not highlighted indicate resistance of the strain; Numbers indicate the MIC [mg/L]. Cut off values were calculated according to EUCAST Clinical Breakpoint Table v. 1.3

	Amp	Cef	Cip	Cot	Gen	Lev	Nit	Tob
P. mirabilis	>200	0,8	<1,3	40/8	<0,25	<2	>260	5
M. morganii	>200	19	<1,3	>160/32	<0,25	<2	>260*	5
P. aeruginosa	>200	>75	5	>160/32	1	16	>260	10
A. faecalis	200	>75	20	>160/32	0,5	8	>260*	20
E. faecalis	100	>75	10	>160/32	1	8	130	<1,25
S. maltophilia	>200	>75	>20	40/8	0,5	>32	>260*	>20
***Myroides* sp.**	>200	>75	10	>160/32	8	16	>260*	>20
S. aureus	6	>75	2,5	40/8	<0,02	4	130*	0,3
E. cloacae	>200	1,2	2,5	20/4	0,06	8	>260*	5

* Weakly sensitive against the solvent

It was shown that antibiotic resistance to gentamycin, tobramycin or vancomycin led to complicated nosocomial infections of enterococci [Gin and Zhanel, 1996; Shadomy and Kirchoff, 1972; Zervos *et al*., 1987]. Surprisingly, *Myroides sp.* A17 was not inhibited by one of the tested antibiotics. In agreement with these findings chromosome-encoded ß-lactamases were reported for the opportunistic pathogens *M. odoratimimus* and *M. odoratus* [Mammeri *et al*., 2002]. In addition, this *M. odoratimimus* isolate showed multiple other antibiotic resistance mechanisms covering a wide range of antibiotics. This is the first description of a biofilm forming, multiresistant, hemolytic, urease, protease and DNase producing *Myroides* strain.

3.2.7 Conclusion

Most bacteria isolated from catheter associated biofilms of patients without obvious symptoms are characterized by a virulent phenotype (exoenzymes, antibiotic resistance) typical for severe CAUTI causing microorganisms. This indicates that those biofilms on catheters from asymptomatic patients represent a reservoir for urinary tract infection causing bacteria.

3.3 Characterization of the isolated *Myroides* sp.

In 2010 a urinary tract catheter originated from a 91 year old female patient was chosen for further microbiological investigation. The catheter was derived from a urological practice, the patient was not hospitalized. This outpatient did not suffer from any symptoms that could be implicated to catheterization and did not receive any antibiotic drugs at this time point.

3.3.1 *Myroides* sp. is a stable part of the catheter biofilm

To isolate microorganisms from the catheter, biofilm mass was scraped from the outer catheter surface suspended in phosphate buffered saline and

plated on tryptone soy agar (Roth, Germany) and MacConkey agar (Difco BD, France). Agar-plates were incubated at 37 °C for 24 h. Single colonies were transferred to fresh agar after 24 h and further incubated. When monoculture purity was reached bacteria were identified by 16S rRNA gene sequencing. Sequences revealed three different species, *Myroides* sp., *Alcaligenes* sp. and *Pantoea* sp.. Moreover, *Kluyvera* sp., *Erwinia* sp. and *Staphylococcus* sp. were identified in another analysis of the same catheter using single strand confirmation polymorphism methodology (chapter 3.1). The *Myroides* isolate was chosen for further investigation. Using the same technique another isolate of *Myroides* sp. (strain A21) was isolated on a urinary tract catheter of the same patient on year later. These findings indicate that the *Myroides* strain persists stable in the microbial community of the urinary tract catheter biofilm.

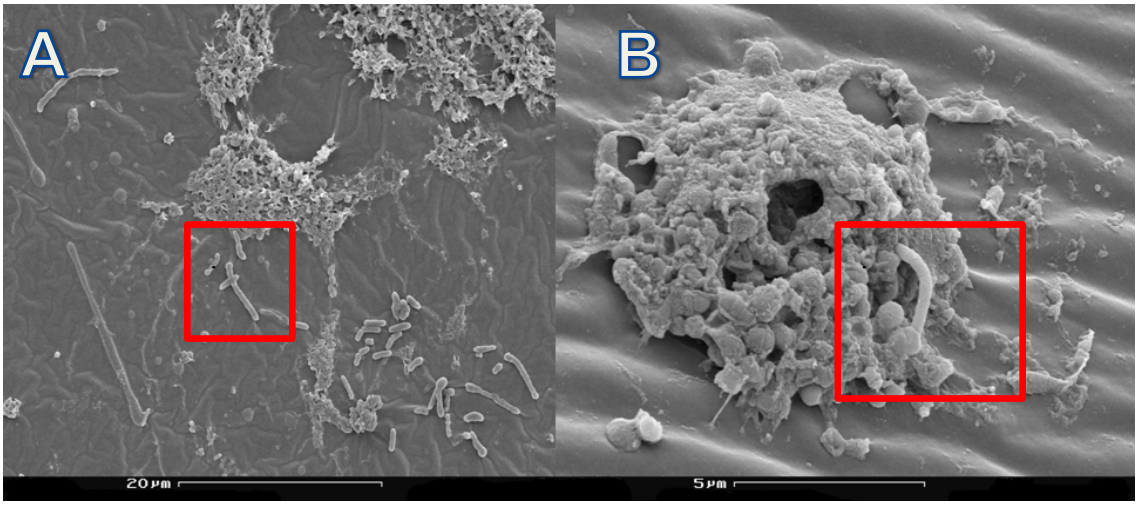

Figure 8: Field emission scanning electron micrographs of the catheter surface. A) overview; B) detail of the biofilm showing bacteria of different shapes embedded in EPS. Electron micrographs were taken by M. Rohde, HZI, Germany. *Myroides* sp. is highlighted in the red frames. Biofilm samples were fixed, dehydrated and critical-point dried. Afterwards they were covered with a gold film by sputter coating and examined in a field emission scanning electron microscope.

To prove that isolate A17 coexists in multibacterial community scanning electron microscope pictures of sections of the catheter were taken. Figure 8 A and B show scanning electron micrographs of the catheter surface. Both pictures show three dimensional biofilm structures of different shaped bacteria and the surrounding extracellular polymeric substances (EPS).

Figure 8 B provides a more detailed view on the biofilm. Small cocci and long rods form a hill shaped piece of biofilm on the surface of the catheter. *Myroides* sp. is highlighted in the frames. Taken together the results indicate that *Myroides* sp. is able to adapt to the urinary tract and there persists in a symbiotic relationship with other bacteria.

3.3.2 *Myroides* sp. A17 cells show uncommon cell features

As 16S rRNA gene sequencing did not yield towards a clear species identification of the isolate A17 it was compared to the clinical relevant and nearest neighbor type strains *M. odoratimimus* (LMG 4029^T) and *M. odoratus* (LMG 1233^T). Therefore, colony and cell morphology was observed. On LB agar plates all strains formed circular, and smooth seamed colonies. Those of isolate A 17 and *M. odoratimimus* were plain and bright yellow while those of *M. odoratus* were convex and dark yellow. In LB broth *Myroides* sp. A17 formed very long cells of up to 60 µm and chains of cells are observed. Similar sizes could be observed for *M. odoratus* while *M. odoratimimus* cells grew up to 4 µm. Figure 9 A-F show scanning electron micrographs of the three strains. Figure 9 A-F show liquid cultures of *Myroides* A17, *M. odoratimimus* (LMG 4029^T) and *M. odoratus* (LMG 1233^T), respectively. All strains were rod shaped, while A17 and *M. odoratus* formed clearly longer cells than *M. odoratimimus*. Figure 9 A-D shows the uncommon cell morphology of the isolate A 17. Cells of the strain grew up to 60 µm (Fig 9 B), chains and formed cap-like structures on the tip of the rod (Fig 9 C). Some were branched (Fig 9 D). None of the type strains showed similar morphology features. Therefore, also the morphological characterization of the isolate indicated strong differences to the type strains and did not yield into a clear species affiliation. The cell sizes of the type strains are in good agreement with literature [Vancanneyt *et al*., 2011]. However, cell sizes of *Myroides* sp. A17 of up to 60 µm are very uncommon. Sizes of prokaryotes range between 0.1 to 750 µm in length [Schulz and Jorgensen, 2001]. Most of these large prokaryotes are cyanobacteria or sulfide oxidizers. The biggest

bacterium known is *Thiomargarita namibiensis*.

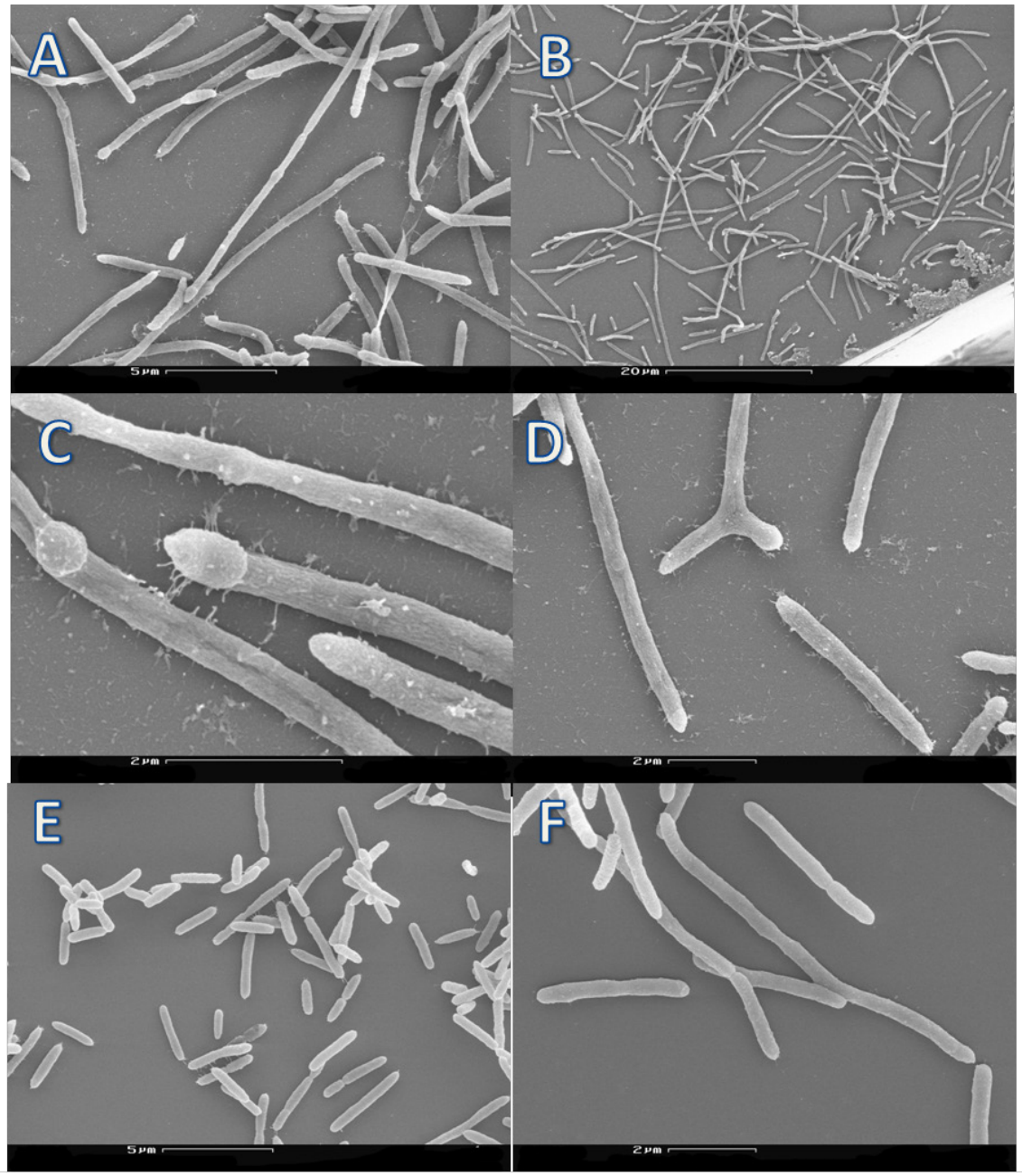

Figure 9: Scanning electron micrographs of the three strains. A) size of Myroides sp. A17, B) single cells and chains, C) cap-like- structures, D) branching at the end of the tips E) *M. odoratimimus* LMG 4029^T, F) *M. odoratus* LMG 1233^T. All strains were grown in liquid medium over night. Samples were processed as described in the material and methods section.

The size of prokaryotic organisms is restricted by the limitations of molecular diffusion of substrates from their environment [Schulz and Jorgensen, 2001]. However, building large cells may lead to competitive advantages. Growing

larger than other bacteria in a microbial community, especially in a biofilm, can lead to a better accession to oxygen or nutrients. As *Myroides* spp. live preferentially aerobic this could be an advantage in this environmental niche.In addition, tip structures of bacterial cells have not been investigated extensively so far. Branching of bacterial cells is mainly known for Cyanobacteria and the Gram-positive Actinobacteria. So called “true branching” is discriminated from “false branching”. False branching means that single cells are divided with by a septum. Branching is there used for morphological taxonomy [Siefert and Fox, 1998]. Interestingly, bacteria of a similar morphology are also present in the SEM pictures of the catheter surface in Figure 8 A and B (chapter 3.3.1). Both pictures show long small rod shaped bacteria . Some of them are branched. These bacteria are supposed to be *Myroides* sp..

In conclusion, the tip structure together with the size and branching events seemed to be unique in the bacterial world.

3.3.3 *Myroides* spp. are ubiquitous distributed in the environment

Since the preferred natural habitat of *Myroides* sp. remains unclear a data base search for *Myroides* sp. nucleotide sequences was performed. For this purpose the 16S rRNA sequence of *M. odoratimimus* LMG 4029^T was uploaded to NCBI BLAST to collect non-identified sequences (similarity more than 97%). Moreover, the NCBI nucleotide collection (http://www.ncbi. nlm.nih.gov/nuccore/) and the ribosomal database project (RDP) (http://rdp.cme.msu.edu/) were screened for all “Myroides”- accessions. The entries for isolation sources were collected.

The NCBI nucleotide collection contained 1034 entries, while 58 entries were found at the RDP collection. Most sequences coded for 16S rRNA genes but some for enzymes e.g. the chromosomal encoded β- lactamases TUS- 1 and MUS- 1 [Mammeri *et al*., 2002].

Most information of isolation sources referred to soil or water related habitats.

Strains were found in soil (NCBI accession number: KC172018.1), composts (AM183094), plant associated in the rhizosphere (GQ383900), on the surface of leafs (GU186112.1) or endophytic (GQ359964.1). Aquatic habitats seem to be sewage (GQ381279.1), rivers (EU311217), river biofilms (GQ398342) as well as marine environments (JF327459). Some strains of *Myroides* seem to be associated with animals as there are for example in the gut of insects (JX457156.1), in the mucus in yellow catfish (GQ359964.1) or on the skin of bats (JF935122). Moreover, *Myroides* was detected in raw chicken meat (JQ178355.1) and on animal skin in a slaughterhouse (EU998999). Remarkably, there are reports about human associated *Myroides* strains. Bacteria were found on the skin of popliteal fossa (HM277221) and volar forearm (JF089226), in urine (JX966100), wounds (JX966100) and the genitourinary tract (JQ259605). Additionally, genomes of each three strains of *M. odoratimimus* derived from wounds and the urogenital tract are sequenced in the NIH Human Microbiom Project.

Myroides species are ubiquitous distributed in the environment. Although, they are sometimes associated with human in health and disease it seems that the natural habitat of the bacteria is soil or water associated. Nevertheless, contamination sources of serious *Myroides* infections remain unclear and further investigations are required. This is particularly true for cases of nosocomial infections for instance the outbreak of urinary tract infections in two hospitals [Ktari *et al*., 2012; Yagci *et al*., 2000].

3.3.4 *Myroides* sp. produces various virulence factors and displays multiple antibiotic resistances

As discussed in chapter 3.2 some extracellular enzymes contribute to the virulence bacteria. Therefore, the *Myroides* strains were tested for production of DNase, urease and hemolysin. The results for *Myroides* A17 are summarized in table 3 and discussed in part 3.2. In contrast to the results obtained for *Myroides* sp. A17 *M. odoratus* LMG 1233^T secreted DNase and urease but no hemolysin. *M. odoratimimus* LMG 4029^T was able do

hydrolyze DNA and to produce hemolysin but no urease production was observed. All strains were non-motile, neither by twitching, swimming or swarming. DNase production of *Myroides* strains has not been tested before. Therefore, this is the first description of *Myroides* strains that secret nucleases which is typical for many bacterial pathogens [Sumby *et al*., 2005]. In contrast there are reports of urease-producing *M. odoratimimus* strains [Vancanneyt *et al*., 2011]. Hydrolysis of urea can raise the pH in the urinary tract and thereby enhance the risk for urinary tract infections (UTI). Productions of urease can lead to selective advantages in the urinary tract towards other bacteria. This fact is important because *Myroides* strains are frequently mentioned in the context of UTI [Ktari *et al*., 2012; Yagci *et al*., 2000]. Similarly, both type strains were described as not hemolytic. This discrepancy can be explained by strain variant behavior of *M. odoratimimus* or different experimental set ups.

The fact that all strains were non-motile is in good agreement with literature [Vancanneyt *et al*., 2011].

Additionally to the antibiotics tested in chapter 3.3, seven more antibiotics were tested to identify an appropriate selection marker for subsequent analysis of *Myroides* sp. A17. Chloramphenicol, streptomycin, carbenicillin, rifampicin, erythromycin, neomycin and spectinomycin were tested in 50 ml/mg step series from concentrations of 50 µg/ml up to 250 µg/ml.

Surprisingly, the isolate was resistant again all tested antibiotics concentrations up to 100 µg/ml. Moreover, all experiments to kill the grown bacterium failed even at high concentrations. Killing the bacterium is essential for quantitative infection assays.

M. odoratimimus antibiotic resistance is referred to be plasmid mediated as well as chromosomal emediated. A study from 2013 from Suganthi and coworkers found that *M. odoratimimus* strains are resistant to amikacin, ampicillin, cefadroxil, cefoperazone, ceftazidine, ceftriaxone, netillin and gentamicin [Suganthi *et al*., 2013]. In addition the two chromosomal encoded β- lactamases TUS-1 and MUS-1 were expressed recombinantly in *E. coli* and studied further [Mammeri *et al*., 2002].

3.3.5 Fatty acid profiling of *Myroides* A17

The analysis of short-chain fatty acids is a chemotaxonomic method for the identification of microorganisms. Usually, the fatty acid components of the cell containing 9 - 20 carbon atoms, hydroxy- and cyclo fatty acids and various isomers are analyzed. The lipids are hydrolyzed under alkaline conditions and esterified with methanol. Thereby, fatty acid methyl esters (FAME) are formed.

The fatty acids methyl esters were separated by gas chromatography, collected in fractions and detected. Subsequent bioinformatic analysis calculated a fatty acid profile with the percentage of each fatty acid, which has been detected.

Table 5: Fatty acid methyl ester derived from Myroides strains. Fatty acids that accounted for less than 1.0% of the total fatty acids in all of the strains studied are not shown. Fatty acid methyl esters are obtained from cultures saponification, methylation and extraction. The fatty acid methyl esters mixtures are separated b gas chromatography and detected by flame ionization.

Taxon	% of total fatty acids											
	13:0 iso	15:0	15:0 iso	15:0 iso 3OH	15:0 anteiso	16:0	16:0 3OH	ECL 16.580 [c]	17:0 iso 3OH	17:1 iso ω9c	Summed feature 4[d]	14:0
Myroides sp. A17	10.0	0	53.6	4.5	Tr	1.5	6.9	0	8.6	5.1	nD	1.4
M. odoratus LMG 1233[Tb]	Tr[e]	Tr	52.1	6.7	2.5	Tr	1.2	1.6	10.8	20.7	Tr	Tr
M. odoratimimus (15 strains)[b]	6.6 ± 1.7	2.1 ± 0.7	45.5 ± 1.3	4.1 ± 0.3	Tr	1.1 0.3	5.2 ± 0.8	Tr	9.7 ± 1.6	10.3 ± 1.7	9.5 ± 1.6	Tr

[a]: nD: not detected
[b]: Data originate from Vancanneyt, Segers, Torck *et al*., 1996
[c]: ECL, equivalent chain length. The identity of the fatty acid is not known.
[d]: Summed feature 4 consisted of one or more of the following fatty acids which could not be separated by the Microbial Identification System: 15:0 iso, 16:1 ω7c and 16:1 ω7t.
[e]:Tr, trace (less than 1.0%)

The fatty acid profile of *Myroide*s A17 was compared to those of the both type strains *M. odoratimimus* LMG 4029^T and *M. odoratus* LMG 1233^T determined by Vancanneyt and coworkers [Vancanneyt *et al*., 1996]. Table 5 shows the results of the whole cell fatty acid analysis determined by gas

chromatography and subsequent bioinformatics.

The main components were $C_{15:0}$iso (53.6%) and $C_{13:0}$iso (10.0%). The hydroxylated compounds $C_{17:0}$iso-3-OH and $C_{16:0}$3-OH were also found in considerable amounts. Finding of $C_{15:0}$iso as the major compound is in good agreement with literature for the type strains. However, the second most compound $C_{13:0}$iso was only found in a trace of less than 1% in *M. odoratus*. Similarly, $C_{14:0}$ was found in *Myroides* sp. A17 with 1.4% but not in the type strains. On the other hand summed feature 4 ($C_{15:0}$iso, $C_{16:1}$ω7c and $C_{16:1}$ω7t) which was the third most compound of *M. odoratimimus* was not detected for *Myroides* sp. A17. The Microbial Identification software package MIDI referred the profile of *Myroides* sp. A17 to *M. odoratus* with a similarity index of 0.515. However, due to peculiarities and in the fatty acid composition and amounts of abundance of the main compounds, FAME profiling did also not yield a clear species affiliation either.

3.3.6 *Myroides* sp. A17 adheres to and invades into non-phagocytic cells

In order to determine the virulence of *Myroides* A17 infection assay of HEp-2 cells were performed. Because no antibiotic effective against the strain was found all quantitative invasion assays failed. These quantitative assays measure the efficacy of bacteria to adhere and invade into cells by direct plating of bacteria after appropriate time of infection. However, to differentiate between extracellular and intracellular bacteria the attached bacteria on the outer surface of the cells need to be eliminated by antibiotic treatment. Therefore, as an alternative, double immunofluorescence assays were performed. Bacterial cells were incubated after infection with primary antibody and fluorescence labeled secondary (red) antibodies and afterward the cells were permeabelized. Bacteria were again incubated with primary antibodies and another secondary antibody (green). Since intracellular bacteria were only stained in green discrimination between intra- and extracellular bacteria is possible. Figure 10 shows the results of double

immunofluorescence assay. Cells were additionally stained with the cytoskeleton dye Alexa Fluor 568 Phalloidin. F-Actin Filaments of the Cytoskeleton are stained in red, intracellular bacteria in green. Figures 10C and 10F show the merges of the two series. Both pictures revealed that *Myroides* sp A17 invaded into the cells.

In order to validate these results electron microscopic studies were performed. Therefore, HEp-2 cells were infected and processed as described in the material and methods section.

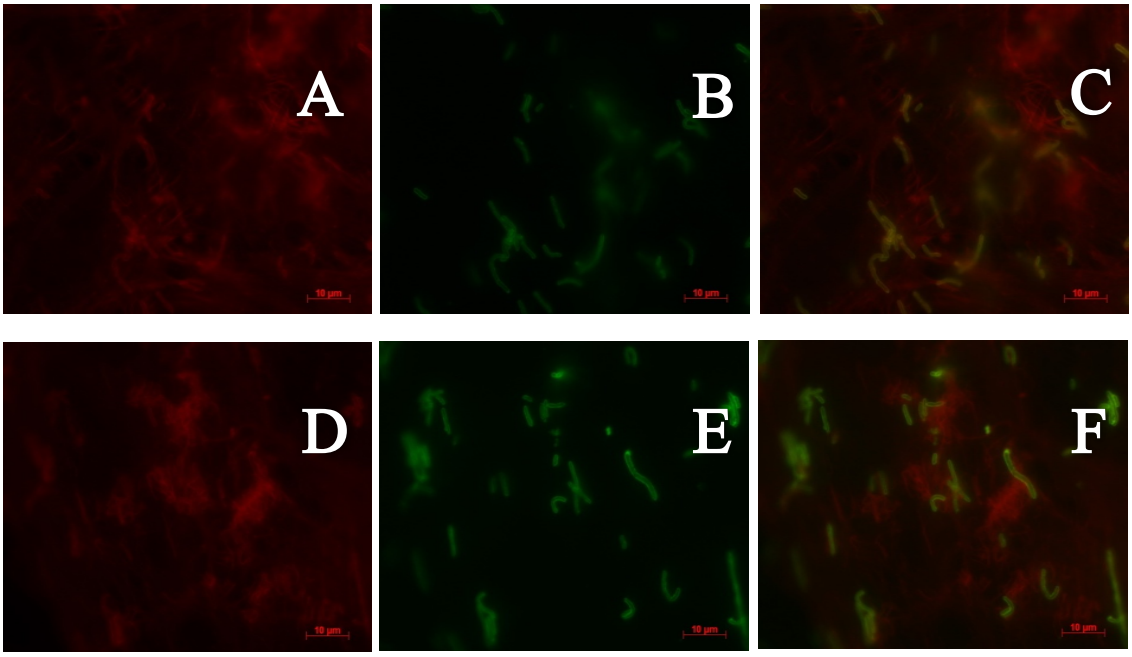

Figure 10: Invasion of *Myroides* sp. into Hep-2 cells. Double immunofluorescence assays were carried out as described in the material and methods section. A) and D): Cytoskeleton stained with Alexa Fluor 568 Phalloidin. B) and E) *Myroides* sp. cells stained with Alexa Fluor 488-labeled antibody. C) and F) merge

Figures 11 shows the results of Field emission scanning electron microscopy (FESEM) (C and D) and transmission electron microscopy (TEM) (A and B). Figure 11 C and D show the surface of the HEp-2 cell and bacteria that are currently invading into the cell. Membrane ruffles (arrow) that incorporated the bacteria were formed.

Ultrathin sections of infected cells (Figure 11 A and B) confirmed the previous infection studies. *Myroides* cells were present in a vacuole. In Figure 11A membrane ruffles were currently enclosing the bacterium. The invasion

process involves two steps: attachment to the host cell surface, followed by internalization into the host cell. [Tang *et al*., 1993]. The process starts with binding of bacterial surface proteins to host receptor molecules. This interaction induces several signal transduction pathways that lead to a local cytoskeleton rearrangement and initiate invasion process [Dersch, 2003]. In principle, there are two different ways for bacteria to enter the eukaryotic cell: “Zippering” and “Triggering” [Dersch, 2003].

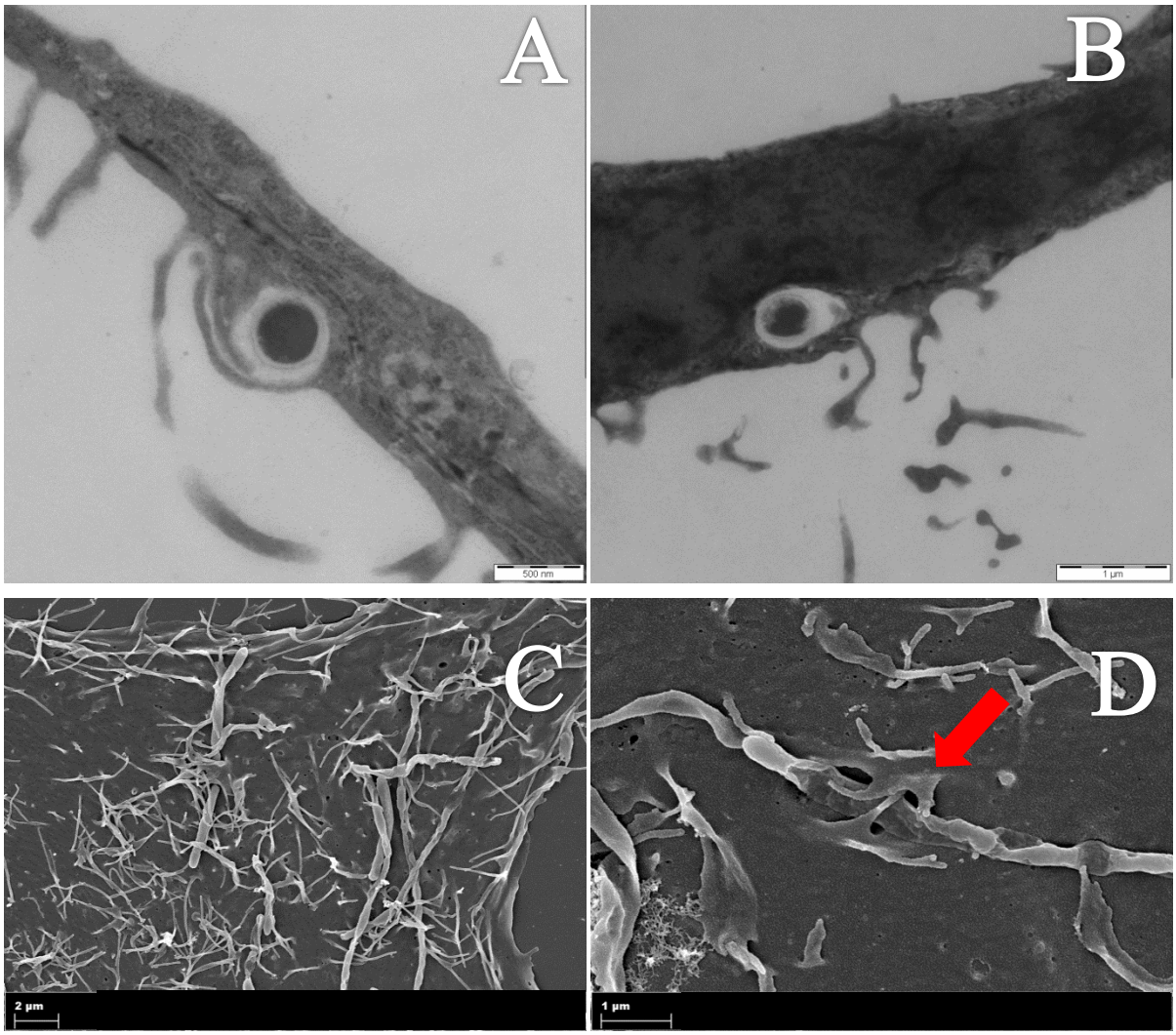

Figure 11: Validation of the invasion of *Myroides* sp. into Hep-2 cells by electron microscopy. Hep- 2 cells were infected and processed as described in chapter 2.7.2**.** Infected cells were analyzed by transmission electron microscopy (A and B) and Field emission scanning electron microscopy (C and D), respectively. Membrane ruffles are formed (arrow).

Regarding zippering the adhesion to the cell leads to receptor clustering. The

bacteria are in close contact to the cell membrane. The formation of ligand-receptor formation induces actin polymerization and membrane extensions. Pseudopod- similar extensions surround the bacterium and after re-closure of the membrane and actin depolymerization the bacterium is enclosed in a vacuole [Cossart and Sansonetti, 2004].

Triggering on the other hand starts with a comparatively loose attachment of the bacterium to the eukaryotic cell. Effector molecules are secreted *via* the Type III secretion system to the host cell and there induce the polymerization process of actin filaments [Dersch, 2003]. As a consequence membrane ruffles are formed and incorporate the bacteria.

Electron microscopic studies revealed that *Myroides* A17 enters the eukaryotic cell *via* the triggering mechanism. This mechanism is well known for *Salmonella* spp. and *Shigella* spp. [Parsot, 2005]. Interestingly, until now there are no reports about intracellular *Myroides* spp. and so far nothing is known about invasion strategies of this species.

3.3.7 Volatile organic compounds analysis are accountable for fruity odor and antimicrobial and antifungal properties

In order to identify the VOCs released by *Myroides* sp. A17, *M. odoratus* and *M. odoratimimus* the closed-loop-stripping analysis was applied followed by identification of the chemical compounds *via* GC/MS spectrometry in cooperation with the chemistry department of the TU Braunschweig under supervision of Professor Stefan Schulz. Analyses of the three strains were performed to provide a deeper insight into the chemotaxonomic nature of the genus. The results are shown in Figure 12A-C. The main compounds released by *Myroides* sp. A17 were ethyl 2- methylpropanoate, ethyl 3-methylbutanoate, propyl 2- methylpropanoate/ propyl butanoate, benzylaldehyde and benzylacohol. *M. odoratus* released mainly 3-methylbutanol, ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, propyl 2- methylpropanoate/propyl butanoate, dimetylpyrazine, propyl 2-methylbutanoate, benylaldehyde, benzylacohol and

benzyl 2-methylbutanoate, respectively. The main compounds detected from *M. odoratimimus* were dimethyldisulfide, ethyl 2-methylpropanoate, methyl 3-methylbutanoate, ethyl 3-methylbutanoate, isopropyl 3-methylbutanoate, S-methyl 3-methylbutanethioate, propyl 3-methylbutanoate, 3-methylbutyl 2-methylpropanoate, 3-methylbutyl 3-methylbutanoate, furfuryl 3-methylbutanoate, 3-(methylthio)propyl 3-methylbutanoate and Benzyl 3-methylbutanoate, respectively. The broadest spectrum of volatiles was found for *M. odoratimimus*. Only seven compounds were detected for *Myroides* sp. A17. However, some of the detected compounds were described to be released by other organisms. Dimethylsulfide, benzaldehyde and dimethylpyrazine were found in cultured bacterial communities that produced malodors in car evaporation units [Diekmann *et al*., 2012]. Especially the widespread pyrazines are linked strong odor properties in nature [Ledauphin *et al*., 2003; Osorio *et al*., 2006]. However, the most released compounds from *Myroides* sp. A17 (ethyl-2-methyl propionate) and *M. odoratus* (ethyl-2-methyl butanoate) were found to produce a sweet-fruity and fruity odor similar to the findings of VOCs produced in pineapple bread [Ledauphin *et al*., 2003; Osorio *et al*., 2006]. Interestingly, one of the compounds found to be released by *M. odoratimimus* was also found in marine bacteria and characterized for its biological effects [Dickschat *et al*., 2005]. The furfuryl 3-methylbutanoate was found to have a high cytotoxic activity against L-929 mouse fibroblasts [Schulz *et al*., 2010]Moreover, it inhibited growth of *Aspergillus fumigatus*; *Botrytis cinerea*; *Pythium debaryanum*; *Hansenula anomala*; *Saccharomyces cerevisiae*, *E. coli tolC* mutant; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; *Micrococcus luteus* and *Mycobacterium phlei* in agar diffusion inhibition assays [Dickschat *et al*., 2005]. The entire conducted analyses strongly suggest that the Myroides A17 isolate represents a new species. However, further experiments have to confirm this hypothesis.

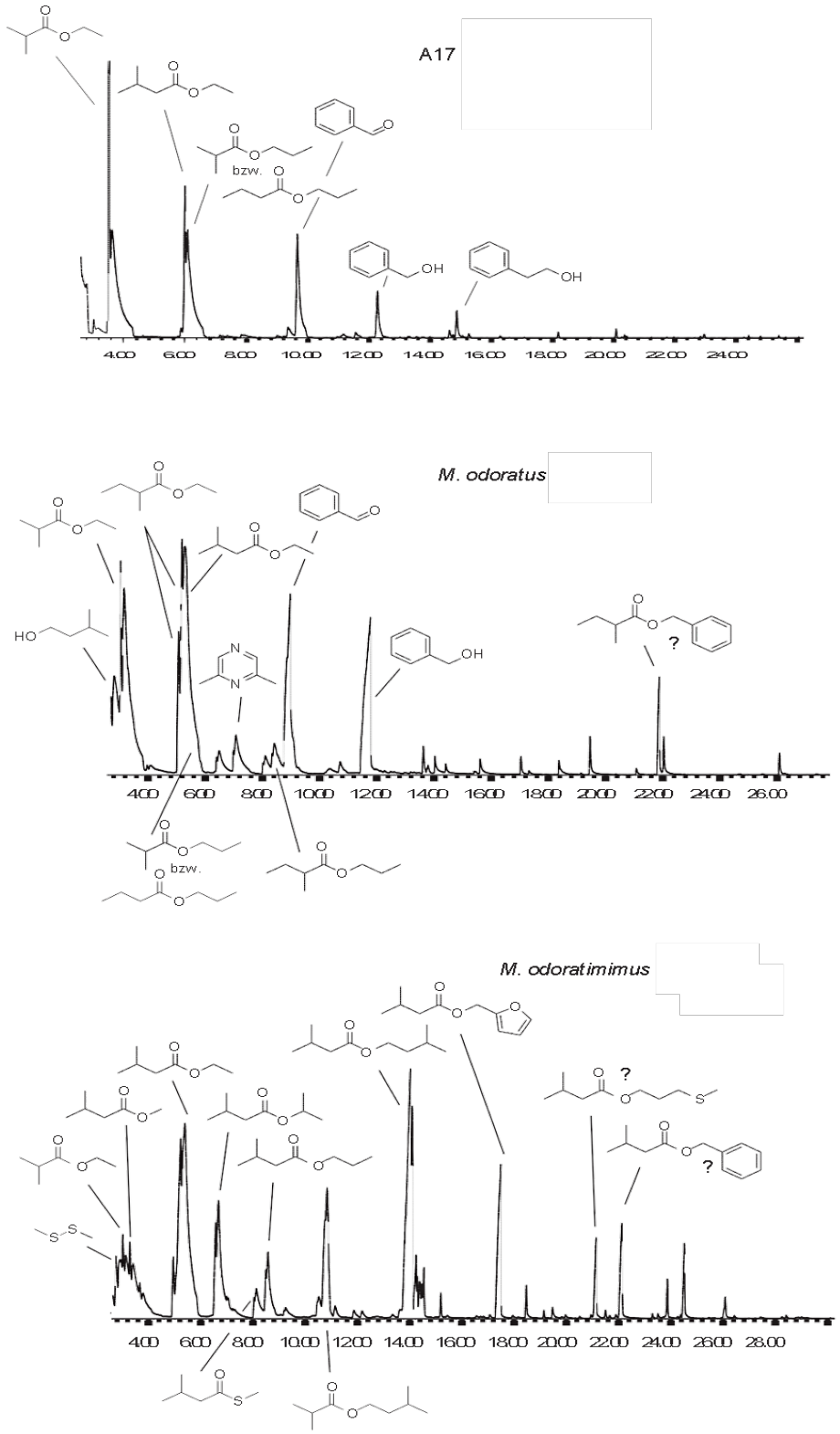

Figure 12 A-C: Volatile organic compound analyses of the three Myroides strains. Spectra of chemical compounds were identified by Closed-loop-stripping analysis and subsequent GC/MS spectrometry for *Myroides* sp. A17 (A), *M. odoratus* (B) and *M. odoratimimus* (C).. Therefore, The volatiles were adsorbed on charcoal and eluted with dichloromethane. The resulting solutions were analyzed by GC/MS.

3.3.8 Genome sequencing of *Myroides* sp. A17

To get a deeper insight into the biology of *Myroides* sp. genome sequences of the strains A17 and 21 were conducted in cooperation with Dr. Sonja Voget and PD Dr. Rolf Daniel from the Göttingen Genome Laboratory (Georg- August- Universität Göttingen). Sequencing of *Myroides* sp. A17 genome was performed using 454 technology and subsequent paired- end and illumina sequencing. Currently, the number of scaffolds was reduced to 34. First results showed that the genome of *Myroides* sp. A17 has a size of 4.3 Mb with 3978. The genome sequence of *Myroides* sp. A21 was revealed by illumina sequencing (288 scaffolds). Interestingly, the size of the genome (3.7 Mb) and the number of estimated gene (3600 counts) is significantly reduced compared to strain A17. Since A21 was isolated one year later than A17 this might point out an adaptation to the environmental niche which results into loss of gene that are not essential for the biofilm living in the urinary tract. Of course, this hypothesis has to be proven again when both genome sequencing projects are finished, genome sequences are closed and annotation of genes is reliable. Nevertheless, the first hints of genome sequencing promise more interesting insights into the biology of the uncommon isolates of *Myroides* sp.

4. Summary

Urinary tract catheters from patients without symptoms are usually contaminated within days after application by microbial biofilms. To investigate the potential of these biofilms to cause severe urinary tract infections 92 catheters were investigated for their microbial community composition using single strand confirmation polymorphism analyses. Five representative communities were investigated further. Three catheters were dominated each by one species and two were co-colonized in almost equally by *Proteus* sp. /*Enterococcus* sp. and *Pseudomonas* sp./*Enterococcus* sp., respectively. The results were confirmed by metaproteomics. This work provides the first culture-independent analysis linking structure of the bacterial community on urinary tract catheters to metaproteomic approach.

Additionally, nine different strains were characterized with regard to their biofilm formation and extracellular enzyme production All isolates formed biofilms with *Stenotrophomonas maltophilia* and *Enterococcus faecalis* showing the strongest biofilm formation. Extracellular enzymes were detected for almost all species. Hemolysis was only found for *Pseudomonas aeruginosa*, *S. maltophilia* and *Myroides* sp.. For eight antibiotics the minimum inhibitory concentrations were determined. Only gentamicin abolished growth on eight out of nine isolates while several other antibiotics showed almost no effect.

Finally, the isolated Myroides strain was subjected to further characterization. Almost nothing is known about virulence or host adaptations of *Myroides* spp. The uncommon isolate of the genus *Myroides* isolated from a catheter biofilm is able to adhere to and invades into human cell line HEp-2. SEM micrographs unraveled uncommon cell morphology with branching rods and cap-like structures on the tip of the cells. Single cells grow up to 60 µm in nutrient broth. Polyphasic taxonomic analyses did not yield a clearly species affiliation. A genome project was almost finished.

In summary, molecular and microbiological characterization of catheter biofilms bacteria from asymptomatic patients revealed their potential to cause severe urinary tract infections.

5. Outlook

1. To validate the conclusion that Gram-positive and Gram-negative interact mutualistic in the biofilm and thereby build a stable community, functional analyses of the proteome data are mandatory. First interpretations of the data set seem to validate this hypothesis. Of course, more samples need to be observed to provide a statistical proof.

2. As described in the result section the isolates harbor a potential reservoir for severe infections and for this purpose the switch from asymptomatic to symptomatic infection needs to be investigated. Metatranscriptomic analysis can be performed for isolates as well as for urinary tract catheter biofilms. Moreover, the culture collection needs to be expanded to other species, for example *E. coli* and the oblige anaerobes *Bacteroides* and *Prevotella* sp. and isolates from nosocomial and infected patients. Infection studies of the isolates will provide a deeper insight to the pathogenic phenotypes. First studies showed that all isolates adhered to bladder epithelial cell line ECV304 and some also invade.

3. To characterize the isolate A17 further genome sequencing was performed. This data will offer more information about the genetic differences between the 3 species and thereby provide a wide set of genetic and molecular biology techniques that can be applied. Potential adhesins and inavsins can be characterized. Moreover, transcriptome analysis will elucidate the adaptations to the urinary tract. Further infection assays including macrophage survival studies will clarify the potential risk for life-threatening infection and host immune response evasion. These studies will also include the role of volatile organic compound, especially the furfuryl ester in infection process. The final species affiliation should be investigated by genome analysis and DNA-DNA hybridization studies. There are a lot of evidences that *Myroides* sp. A17 belongs to a so far unknown species.

6. References

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8. Appendices

Table 1 appendix: list of catheters with patients data. Listed are catheter numbers, type, patients ID and sex of the patient. The fifth column shows the isolates from the corresponding catheters that were characterized further in this study.

number	type	patient	sex	Isolate
1.1 (**patient 1**)	Suprapubic	SchH6529	m	-
1.2	Suprapubic	SchH6529	m	-
2.1	Transurethral	OW13138	m	-
2.2	Transurethral	OW13138	m	-
3.1 (**patient 2**)	Suprapubic	GJ13729.	m	-
3.2	Suprapubic	GJ13729.	m	-
3.3	Suprapubic	GJ13729.	m	-
4.1 (**patient 3**)	Suprapubic	WE7522.	f	**Isolate *Staphylococcus aureus* A 22**
4.2	Suprapubic	WE7522	f	-
5.1	-	-	-	-
6.1	-	-	-	-
7.1	-	-	-	-
8.1	Suprapubic	HE7819	f	-
8.2	Suprapubic	HE7819	f	-
9.1	Suprapubic	WM51056	f	-
9.2	Suprapubic	WM51056	f	-
9.3	Suprapubic	WM51056	f	-
10.1 (**patient 4**)	Suprapubic	MA1362	m	-
10.2	Suprapubic	MA1362	m	-
10.3	Suprapubic	MA1362	m	-
11.1	Suprapubic	BF30630	m	-
12.1	Transurethral	BA13424	m	-
12.2	Transurethral	BA13424	m	-
12.3	Transurethral	BA13424	m	-
13.1	-	RG22922	-	-
13.2	-	RG22922	-	-
14.1	Suprapubic	KR12926	m	**Isolate *A. faecalis* A7**
14.2	Suprapubic	KR12926	m	-
14.3	Suprapubic	KR12926	m	-
15.1	Suprapubic	HaHel23340	m	-
15.2	Suprapubic	HaHe23340l	m	-
15.3	Suprapubic	HaHel23340	m	-
16.1	Suprapubic	KE14723	f	-
16.2	Suprapubic	KE14723	f	-
16.3	Suprapubic	KE14723	f	-
17.1 (**patient 5**)	Suprapubic	H.H12236	m	**Isolate *P. mirabilis* A1**
17.2	Suprapubic	HH12223	m	-
18.1	Suprapubic	BI30763	f	-

Table 1 appendix, continued

18.2	Suprapubic	BI30763	f	-
18.3	Suprapubic	BI30763	f	-
19.1	Suprapubic	SH21441	m	-
19.2	Suprapubic	SH21441	m	-
19.3	Suprapubic	SH21441	m	-
20.1	Suprapubic	PG30732	m	-
20.2	Suprapubic	PG30732	m	-
20.3	Suprapubic	PG30732	m	-
21.1	Suprapubic	SE22235	m	-
21.2	Suprapubic	SE22235	m	-
22.1	Suprapubic	LP14460	f	-
22.2	Suprapubic	LP14460	f	-
23.1	Suprapubic	AY311248	m	-
24.1	Suprapubic	GH201028	m	-
24.2	Suprapubic	GH201028	m	**Isolate *S. maltophilia* A14**
25.1	Suprapubic	PW111033	m	-
26.1		SH27919		-
27.1	Suprapubic	ER251055	f	-
27.2	Suprapubic	ER251055	f	-
28.1	Suprapubic	GA251114	m	-
28.2	Suprapubic	GA251114	m	-
29.1	-	SG141028	-	-
30.1	-	DE221019	-	-
30.2	-	DE221019	-	**Isolate *Myroides* sp. A17**
30.3	-	DE221019	-	-
31.1	-	PK81126	-	-
31.2	-	PK81126.	-	-
32.1	-	SH6529	-	-
33.1	-	WE7522	-	-
34.1	-	RG22929	-	-
35.1	-	KH3336	-	-
36.1	Transurethral	VR161222	m	-
36.2	Transurethral	VR161222	m	-
37.1	-	GA15751	-	-
38.1	-	SchmW13342	-	-
39.1	-	BH17903	-	-
39.2	-	BH17903	-	-
40.1	-	ME7224	-	-
40.2	-	ME7224	-	**Isolate *M. morganii* A2**
41.1	-	OW281130	-	-
42.1	-	SchE	-	-
43.1	Suprapubic	AH8239	f	-
44.1	-	WG121028	-	-
45.1	Suprapubic	FH28123	m	-
46.1	Transurethral	KH16520	m	-
46.2	Transurethral	KH16520	m	**Isolates *P. aeruginosa* A6, *E. faecalis* A8**
47.1	-	GF8424	-	-
48.1	-	GB14437	-	-

Table 1 appendix, continued

49.1	-	WM51056	-	-
50.1	Suprapubic	RG21945	m	-
50.2	Suprapubic	RG21945	m	-
50.3	Suprapubic	RG21945	m	-
51.1	Transurethral	GH28123	m	-
52.1	-	AH2839	-	-

Table 2 appendix: Results of the SSCP sequencing analyses. First colum contains the number of the corresonding catheter. The third character indicates the SSCP sample number of the corresponigg catheter sample. The second coloumn reveals the nearest neighbour (type strains with NCBI accession number) obtained by sequence analyses

SSCP sample number	**Nearest neighbor**
1.1.1 (**patient 1**)	*Proteus mirabilis* ATCC 29906(T)
1.1.2	*Pseudomonas aeruginosa* LMG 1242(T)
1.1.3	*Pseudomonas aeruginosa* LMG 1242(T)
1.1.4	*Aerococcus sanguinicola* CCUG 43001(T)
1.1.6	*Aerococcus urinae* NCFB 2893(T)
2.1.1	*Escherichia coli* O157 EC4115
2.1.2	*Enterococcus faecalis* V583
2.1.3	*Enterococcus faecalis* V583
2.1.4	*Aerococcus urinae* NCFB 2893(T)
2.1.5	*Aerococcus urinae* NCFB 2893(T)
2.1.6	*Klebsiella pneumoniae* ATCC 13884(T)
2.1.7	*Aerococcus urinae* NCFB 2893(T)
3.1.1 (**patient 2**)	*Enterococcus faecalis* V583
3.1.2	*Aerococcus urinae* NCFB 2893(T)
3.1.3	*Aerococcus urinae* NCFB 2893(T)
3.1.4	*Aerococcus urinae* NCFB 2893(T)
3.1.5	*Aerococcus urinae* NCFB 2893(T)
4.1.1 (**patient 3**)	*Enterococcus faecalis* V583
4.1.2	*Stenotrophomonas maltophilia* ATCC 19861(T)
4.1.3	*Escherichia coli* O157 EC4115
4.1.4	*Corynebacterium striatum* ATCC 6940(T)
4.1.5	*Pseudomonas aeruginosa* LMG 1242(T)
4.1.6	*Enterococcus faecalis* V583
4.1.8	*Aerococcus urinae* NCFB 2893(T)
4.1.9	*Enterococcus villorum* LMG 12287(T)
4.1.10	*Aerococcus urinae* NCFB 2893(T)
5.1.1	*Bacteroides fragilis* NCTC 9343(T)
5.1.2	*Staphylococcus warneri* ATCC 27836(T)
5.1.3	*Morganella morganii* subsp. *sibonii* DSM 14850(T)
5.1.4	*Corynebacterium striatum* ATCC 6940(T)
5.1.5	*Morganella morganii* subsp. *sibonii* DSM 14850(T)
6.1.1	*Alcaligenes aquatilis* LMG 22996(T)
6.1.2	*Proteus mirabilis* ATCC 29906(T)
6.1.3	*Enterococcus faecalis* V583
6.1.4	*Aerococcus urinae* NCFB 2893(T)
7.1.1	*Escherichia coli* O157 EC4115
7.1.2	*Enterococcus faecalis* V583
7.1.3	*Aerococcus urinae* NCFB 2893(T)
8.1.1	*Klebsiella variicola* F2R9(T)
8.1.2	*Proteus mirabilis* ATCC 29906(T)
8.1.3	*Morganella morganii* subsp. *sibonii* DSM 14850(T)

table 2 appendix, continued

8.1.4	*Morganella morganii* subsp. *sibonii* DSM 14850(T)
8.1.5	*Corynebacterium striatum* ATCC 6940(T)
9.1.1	*Proteus mirabilis* ATCC 29906(T)
9.1.2	*Streptococcus agalactiae* NCTC 8181(T)
9.1.3	*Enterococcus faecalis* V583
9.1.5	*Vagococcus carniphilus* ATCC BAA-640(T)
9.1.6	*Aerococcus urinae* NCFB 2893(T)
9.1.7	*Aerococcus urinae* NCFB 2893(T)
9.1.8	*Aerococcus urinae* NCFB 2893(T)
9.1.9	*Aerococcus urinae* NCFB 2893(T)
9.2.1	*Alcaligenes aquatilis* LMG 22996(T)
9.2.2	*Staphylococcus warneri* ATCC 27836(T)
9.2.3	*Morganella morganii* subsp. *sibonii* DSM 14850(T)
9.2.4	*Streptococcus intermedius* ATCC 27335(T)
9.2.5	*Campylobacter ureolyticus* ATCC 33387(T)
9.2.6	*Aerococcus urinae* NCFB 2893(T)
9.2.7	*Aerococcus urinae* NCFB 2893(T)
9.2.8	*Parvimonas micra* ATCC 33270(T)
10.1.1 (**patient 4**)	*Alcaligenes aquatilis* LMG 22996(T)
10.1.2	*Proteus mirabilis* ATCC 29906(T)
10.1.3	*Proteus mirabilis* ATCC 29906(T)
10.1.4	*Escherichia coli* O157 EC4115
10.1.5	*Corynebacterium striatum* ATCC 6940(T)
10.1.6	*Granulicatella elegans* DSM 11693(T)
10.1.7	*Streptococcus agalactiae* NCTC 8181(T)
10.1.8	*Enterococcus faecalis* V583
11.1.1	*Bacteroides fragilis* NCTC 9343(T)
11.1.2	*Proteus mirabilis* ATCC 29906(T)
11.1.3	*Proteus mirabilis* ATCC 29906(T)
11.1.4	*Proteus mirabilis* ATCC 29906(T)
11.1.5	*Streptococcus agalactiae* NCTC 8181(T)
11.1.6	*Proteus mirabilis* ATCC 29906(T)
12.1.1	*Bacteroides fragilis* NCTC 9343(T)
12.1.2	*Stenotrophomonas maltophilia* ATCC 19861(T)
12.1.3	*Streptococcus intermedius* ATCC 27335(T)
12.1.4	*Streptococcus agalactiae* NCTC 8181(T)
12.1.5	*Vagococcus carniphilus* ATCC BAA-640(T)
12.1.6	*Aerococcus urinae* NCFB 2893(T)
13.1.1	*Stenotrophomonas maltophilia* ATCC 19861(T)
13.1.2	*Enterococcus faecalis* V583
13.1.3	*Aerococcus urinae* NCFB 2893(T)
13.1.4	*Klebsiella pneumoniae* ATCC 13884(T)
13.2.1	*Klebsiella pneumoniae* ATCC 13884(T)
13.2.2	*Enterobacter aerogenes* NCTC 10006(T)
13.2.3	*Staphylococcus warneri* ATCC 27836(T)
13.2.4	*Morganella morganii* subsp. *sibonii* DSM 14850(T)
13.2.5	*Enterococcus faecalis* V583
13.2.6	*Aerococcus urinae* NCFB 2893(T)

table 2 appendix, continued

13.2.7	*Klebsiella pneumoniae* ATCC 13884(T)
13.2.8	*Lactobacillus psittaci* CCUG 42378(T)
14.1.1	*Paenalcaligenes hominis* CCUG 53761A(T)
14.1.2	*Alcaligenes aquatilis* LMG 22996(T)
14.1.3	*Proteus mirabilis* ATCC 29906(T)
14.1.4	*Stenotrophomonas maltophilia* ATCC 19861(T)
14.1.5	*Proteus mirabilis* ATCC 29906(T)
14.1.6	*Proteus mirabilis* ATCC 29906(T)
14.1.7	*Morganella morganii* subsp. *sibonii* DSM 14850(T)
14.1.8	*Morganella morganii* subsp. *sibonii* DSM 14850(T)
15.1.1	*Actinobaculum schaalii* CCUG 27420(T)
15.1.2	*Morganella morganii* subsp. *sibonii* DSM 14850(T)
15.1.3	*Corynebacterium striatum* ATCC 6940(T)
15.1.4	*Enterococcus faecalis* V583
15.1.5	*Aerococcus urinae* NCFB 2893(T)
16.1.1	*Klebsiella pneumoniae* ATCC 13884(T)
16.1.2	*Klebsiella pneumoniae* ATCC 13884(T)
16.1.3	*Aerococcus urinae* NCFB 2893(T)
17.1.1 (**patient 5**)	*Stenotrophomonas maltophilia* ATCC 19861(T)
17.1.2	*Proteus mirabilis* ATCC 29906(T)
17.1.3	*Morganella morganii* subsp. *sibonii* DSM 14850(T)
17.1.4	*Streptococcus agalactiae* NCTC 8181(T)
17.1.5	*Enterococcus faecalis* V583
17.1.6	*Aerococcus urinae* NCFB 2893(T)
17.1.7	*Aerococcus urinae* NCFB 2893(T)
17.1.8	*Aerococcus urinae* NCFB 2893(T)
17.1.9	*Aerococcus urinae* NCFB 2893(T)
18.1.1	*Alcaligenes aquatilis* LMG 22996(T)
18.1.2	*Morganella morganii* subsp. *sibonii* DSM 14850(T)
18.1.3	*Tissierella praeacuta* ATCC 25539(T)
18.1.4	*Aerococcus urinae* NCFB 2893(T)
18.1.5	*Aerococcus urinae* NCFB 2893(T)
18.1.6	*Aerococcus urinae* NCFB 2893(T)
18.1.7	*Aerococcus urinae* NCFB 2893(T)
18.1.8	*Aerococcus urinae* NCFB 2893(T)
19.1.1	*Prevotella buccalis* ATCC 35310(T)
19.1.2	*Morganella morganii* subsp. *sibonii* DSM 14850(T)
19.1.3	*Aerococcus urinae* NCFB 2893(T)
20.1.2	*Streptococcus agalactiae* NCTC 8181(T)
20.1.3	*Enterococcus faecalis* V583
20.1.4	*Aerococcus urinae* NCFB 2893(T)
20.1.5	*Aerococcus urinae* NCFB 2893(T)
20.1.6	*Aerococcus urinae* NCFB 2893(T)
21.1.1	*Stenotrophomonas maltophilia* ATCC 19861(T)
21.1.2	*Corynebacterium striatum* ATCC 6940(T)
22.1.1	*Alcaligenes aquatilis* LMG 22996(T)
22.1.2	*Staphylococcus warneri* ATCC 27836(T)
22.1.3	*Enterococcus faecalis* V583

table 2 appendix, continued

23.1.1	*Proteus mirabilis* ATCC 29906(T)
23.1.2	*Escherichia coli* O157 EC4115
23.1.3	*Aerococcus sanguinicola* CCUG 43001(T)
23.1.4	*Enterococcus faecalis* V583
23.1.6	*Peptoniphilus harei* DSM 10020(T)
24.1.1	*Bacteroides fragilis* NCTC 9343(T)
24.1.2	*Alcaligenes aquatilis* LMG 22996(T)
24.1.3	*Stenotrophomonas maltophilia* ATCC 19861(T)
24.1.4	*Staphylococcus warneri* ATCC 27836(T)
24.1.5	*Enterococcus faecalis* V583
25.1.1	*Proteus mirabilis* ATCC 29906(T)
25.1.2	*Escherichia coli* O157 EC4115
25.1.3	*Enterococcus faecalis* V583
1.2.1	*Citrobacter koseri*
1.2.2	*Actinobaculum schaalii*
1.2.3	*Enterococcus faecalis*
2.2.10	*Enterococcus faecalis* V583 AE016830
2.2.11	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
2.2.12	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
2.2.15	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
2.2.16	*Enterococcus faecalis* V583 AE016830
2.2.2	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
2.2.4	*Actinobaculum schaalii* CCUG 27420(T) (Y12329)
2.2.5	*Enterococcus faecalis* V583 (AE016830)
2.2.6	*Enterococcus faecalis* V583 (AE016830)
2.2.7	*Enterococcus villorum* LMG 12287(T) (AJ271329)
2.2.8	*Enterococcus faecalis* V583 (AE016830)
2.2.9	*Melissococcus plutonius* NCDO 2443(T)(X75751)
3.2.1	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.10	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.11	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.12	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.13	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.14	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.15	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.17	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.18	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.19	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.20	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.21	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.3	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
3.2.4	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.5	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.6	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.7	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.8	*Aerococcus urinae* NCFB 2893(T) (M77819)
3.2.9	*Aerococcus urinae* NCFB 2893(T) (M77819)
4.2.1	*Bacteroides fragilis*

table 2 appendix, continued

4.2.2	*Prevotella timonensis*
4.2.4	*Parvimonas micra*
4.2.5	*Veillonella dispar*
4.2.6	*Peptoniphilus harei*
4.2.7	*Aerococcus urinae*
4.2.8	*Aerococcus urinae*
8.2.1	*Klebsiella pneumoniae* ATCC 13884(T) (Y17657)
8.2.10	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
8.2.11	*Aerococcus urinae* NCFB 2893(T) (M77819)
8.2.12	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
8.2.13	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
8.2.14	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
8.2.15	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
8.2.2	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
8.2.3	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
8.2.4	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
8.2.5	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
8.2.6	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
8.2.7	*Corynebacterium striatum* ATCC 6940(T)(ACGE01000001)
8.2.8	*Corynebacterium striatum* ATCC 6940(T)(ACGE01000001)
8.2.9	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
17.2.1	*Staphylococcus* sp.
17.2.2	*Oligella urethralis*
17.2.4	*Providencia vermicola*
24.2.1	*Alcaligenes faecalis subsp. faecalis*
24.2.2.	*Proteus mirabilis*
24.2.3	*Enterococcus faecalis*
24.2.5	*Aerococcus urinae*
24.2.6	*Aerococcus urinae*
30.2.1	*Kluyvera intermedia*
30.2.2	*Kluyvera intermedia*
30.2.3	*Erwinia persicina*
30.2.4	*Kluyvera intermedia*
30.2.5	*Staphylococcus* sp.
30.2.6	*Kluyvera intermedia*
39.2.1	*Staphylococcus* sp.
39.2.2	*Staphylococcus* sp.
39.2.3	*Staphylococcus* sp.
39.2.4	*Staphylococcus* sp.
39.2.5	*Providencia vermicola*
39.2.6	*Staphylococcus* sp.
39.2.7	*Staphylococcus* sp.
10.2.7	*Aerococcus urinae* NCFB 2893(T) (M77819)
10.2.8	*Aerococcus urinae* NCFB 2893(T) (M77819)
10.2.9	*Aerococcus urinae* NCFB 2893(T) (M77819)
12.2.1	*Bacteroides thetaiotaomicron* VPI-5482(T) (AE015928)
12.2.10	*Aerococcus urinae* NCFB 2893(T) (M77819)
12.2.11	*Aerococcus urinae* NCFB 2893(T) (M77819)

table 2 appendix, continued

12.2.12	*Aerococcus urinae* NCFB 2893(T) (M77819)
12.2.13	*Aerococcus urinae* NCFB 2893(T) (M77819)
12.2.14	*Aerococcus urinae* NCFB 2893(T) (M77819)
12.2.2	*Prevotella timonensis* 4401737(T) (DQ518919)
12.2.3	*Morganella morganii* subsp. *sibonii* DSM 14850(T) (DQ358146)
12.2.4	*Morganella morganii* subsp. *sibonii* DSM 14850(T) (DQ358146)
12.2.5	Candidatus *Peptostreptococcus massiliae* 2002-69396 (AY244772)
12.2.6	*Veillonella dispar* ATCC 17748(T) (ACIK02000021)
12.2.7	*Veillonella dispar* ATCC 17748(T) (ACIK02000021)
12.2.8	*Campylobacter ureolyticus* ATCC 33387(T) (L04321)
12.2.9	*Aerococcus urinae* NCFB 2893(T) (M77819)
13.3.1	*Klebsiella pneumoniae* ATCC 13884(T) (Y17657)
13.3.2	*Klebsiella pneumoniae* ATCC 13884(T) (Y17657)
13.3.3	*Klebsiella pneumoniae* ATCC 13884(T) (Y17657)
13.3.4	*Escherichia coli* O157 EC4115 (CP001164)
14.3.1	*Xanthomonas codiaei*
14.3.2	*Staphylococcus sp.*
14.3.3	*Aerococcus sanguinicola*
14.3.4	*Aerococcus sanguinicola*
14.3.5	*Aerococcus sanguinicola*
14.3.6	*Aerococcus sanguinicola*
14.3.7	*Aerococcus sanguinicola*
9.3.1	*Aerococcus urinae* NCFB 2893(T) (M77819)
9.3.10	*Aerococcus urinae* NCFB 2893(T) (M77819)
9.3.12	*Aerococcus urinae* NCFB 2893(T) (M77819)
9.3.13	*Aerococcus urinae* NCFB 2893(T) (M77819)
9.3.14	*Aerococcus urinae* NCFB 2893(T) (M77819)
9.3.15	*Aerococcus urinae* NCFB 2893(T) (M77819)
9.3.16	*Aerococcus urinae* NCFB 2893(T) (M77819)
9.3.17	*Aerococcus urinae* NCFB 2893(T) (M77819)
9.3.2	*Aerococcus urinae* NCFB 2893(T) (M77819)
9.3.3	*Alcaligenes aquatilis* LMG 22996(T) (AJ937889)
9.3.5	*Proteus mirabilis* ATCC 29906(T) (ACLE01000013)
9.3.6	*Actinobaculum schaalii* CCUG 27420(T) (Y12329)
9.3.7	*Actinobaculum schaalii* CCUG 27420(T) (Y12329)
9.3.8	*Enterococcus faecalis* V583 (AE016830)
9.3.9	*Enterococcus faecalis* V583 (AE016830)
10.3.2	*Bacteroides fragilis*
10.3.4	*Morganella morganii*
10.3.5	*Corynebacterium* sp.
10.3.6	*Enterococcus* sp.
14.3.1	*Xanthomonas codiaei*
14.3.2	*Staphylococcus* sp.
14.3.3	*Aerococcus sanguinicola*
14.3.4	*Aerococcus sanguinicola*
14.3.5	*Aerococcus sanguinicola*
14.3.6	*Aerococcus sanguinicola*
14.3.7	*Aerococcus sanguinicola*

table 2 appendix, continued

16.3.1	*Citrobacter koseri*
16.3.2	*Bacteroides fragilis*
16.3.4	*Stenotrophomonas maltophilia*
16.3.5	*Corynebacterium amycolatum*
16.3.6	*Corynebacterium amycolatum*
16.3.7	*Enterococcus faecalis*
16.3.8	*Bacteroides fragilis*